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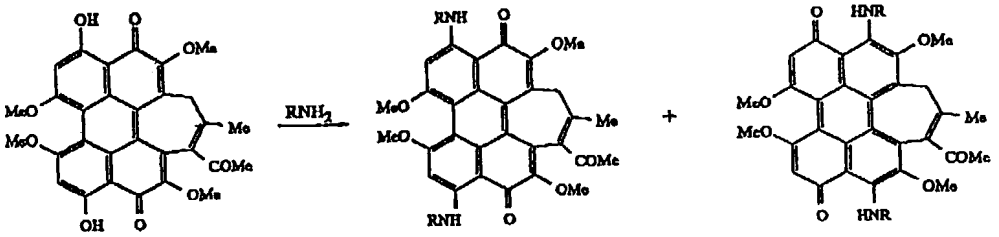

Hypocrellin B | Isomer A | Isomer B

(57) Abstract

The invention involves a method and compositions for use in photodynamic therapy. Novel perylenequinone derivatives, conjugates comprising perylenequinone derivatives and a binding agent, and methods of treatment using these compositions are disclosed.

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SUBSTITUTED PERYLENEQUINONES FOR USE IN PHOTODYNAMIC THERAPY

Technical Field of the Invention

The invention involves a method for using and enhancing the activity of compounds that have anti-cancer activity, anti-viral activity, cosmetic improvement, and/or non-malignant disease activity in animals, including humans. The invention also involves diagnostic methods and compositions.

Background of the Invention

Treatment for cancer has traditionally encompassed three main strategies: surgery, chemotherapy, and radiotherapy. Although considerable progress in these areas has been attained, the search for more effective and safe alternative treatments continues. Lipson, et al. were the first to use photodynamic therapy (PDT), in 1966 at the Mayo Clinic [*Proc. IX Internat. Cancer Congress*, page 393 (1966)]. By 1972 widespread interest in PDT had prompted animal experiments, the results of which demonstrated that the combination of a photosensitizer (e.g., fluorescein), light, and cellular oxygen could inhibit tumor growth [Dougherty, T.J., *JNCI* 52:1333 (1974)]. The first animal study using hematoporphyrin derivatives (HpD) was reported in 1975 [Dougherty, et al., *JNCI* 55:115 (1975)].

Since the advent of HpD and its more purified version, Photofrin®, PDT of tumors has progressed to phase III clinical trials. Although HpD has been a useful tissue photosensitizer, problems are associated with its use, including prolonged cutaneous phototoxicity; the compositions are oligomeric mixtures of hydrophilic molecules prone to molecular aggregation (with concomitant loss of photopotentiation); complicated pharmacokinetics; poor absorption and photoactivation in the "therapeutic window" (600 nm to 850 nm, i.e., visible red light). Furthermore, its batch

reproducibility, even the clinical compositions, has been poor.

Interest in PDT to treat human malignancies and non-malignant conditions has increased markedly since the advent and clinical application of hematoporphyrin derivatives. To date PDT of cancers (in both laboratory and clinical trials) has primarily involved the use of porphyrin-based photosensitizers e.g., Photofrin-II®. These compounds have been used, and interest in PDT has increased, despite porphyrin-based photosensitizer's sub-optimal light absorption characteristics, source-dependent biological response and molecular composition, and difficulties associated with prolonged photosensitization of the host.

These limitations have prompted a search for alternative photosensitizers. Current candidates include benzoporphyrin derivative monoacid ring A [Richter, et al., *Photochem. Photobiol.* 52:495 (1990)], and 5-aminolevulinic acid/protoporphyrin IX [Kennedy, et al., *J. Photochem. Photobiol B Biol.* 6(1-2):143 (1990)]. Additional studies include merocyanine 540, phthalocyanine (CASPc), mono-L-aspartyl chlorin e_6 (MACE), Nile blue, anthrapyrazoles, anthracenediones, anthracyclines, and hypocrellins [Diwu, et al., *Photochem. Photobiol.* 52(3):609 (1990)].

The photosensitizing properties of perylenequinoid pigments (PQPs), such as hypocrellins, in biological systems have been recognized during the past two decades. See Diwu, et al., *J. Photochem. Photobiol. A: Chem.*, 64:273 (1992); Zhang et al., (1989); and Wan, et al., "Hypocrellin A, a new drug for photochemotherapy," **Kexue Tongbao** (English edition) 26:1040 (1981).

Hypocrellins derive their name from *Hypocrella bambusae sacc.*, a parasitic fungus of the Sinarundinaria species, which grows abundantly in the northwestern region of the Yunnan Province (People's Republic of China), the southeastern region of Tibet, and certain parts of Sri Lanka. Hypocrellins belong to the general class of perylenequinoid pigments, and include hypocrellin A (HA) and hypocrellin B (HB).

Oral administration of HA causes hypericism, a state of skin sensitivity to visible light. It has now been shown that the photosensitizing effects of HA depend on the presence of oxygen, indicating that naturally occurring HA can be used as a photodynamic agent (Wan, et al., above).

Summary of the Invention

In accordance with the present invention, derivatives of perylenequinone pigments (PQPs) are a new class of photosensitizing agents, and are useful in photodynamic therapy. Moreover, PQP derivatives, particularly functionalized PQPs, may be conjugated to binding agents that bind pre-determined cells or structures *in vitro* or *in vivo*.

The methods and compositions of the present invention provide meaningful improvements over Photofrin®. For example, hypocrellins exhibit substantial absorption in the red spectral region; produce high singlet oxygen yield; can be produced in pure, monomeric form; may be derivatized to optimize properties of red light absorption, tissue biodistribution, and toxicity; have reduced residual cutaneous photosensitivity; and are rapidly excreted. They afford nuclear targeting by covalent attachment to DNA minor-groove binding agents, such as stapled lexitropins, to enhance phototoxicity. They are not genotoxic. This trait is important in the context of treatment-related secondary malignancies. Conjugation with monoclonal antibodies (e.g., immunoconjugates) affords specificity with respect to the treatment of a variety of diseases, including ovarian cancer and breast cancer. Fluorescence properties of the hypocrellins, or the fact that they are strongly colored, facilitates their use in the diagnosis of tumors and metastatic disease, and their spectral fingerprint facilitates differentiation between malignant, normal, inflamed, or physically damaged tissues. Further, these fluorescence properties can be detected through a variety of optical detection means.

Conjugation with monoclonal antibodies also affords a high degree of phototherapeutic specificity for a variety of diseases, including ovarian and breast cancer. For example, HBEA-R1 transcutaneous phototherapy permanently ablates the EMT6/Ed tumor growing in the flank of Balb/c mice, with minimal cutaneous effects. The conjugates mediate phototoxicity through apoptotic cell death, primarily through type II photochemical reactions with intracellular and membrane targets. In the total absence of oxygen, phototoxicity is mediated through the type I photochemical reaction ("radical cascade"). This feature is critical to the management of hypoxic tumor cells,

which currently limit cures by radiation and drugs. They retain significant phototoxicity at 688 nm, well within the phototherapeutic window [Estey, et al., *Cancer Chemother. Pharmacol.*, 37:343 (1996)].

Perylenequinones comprise a growing and highly diverse group of natural pigments, and they posses some unique chemical and biological properties. The natural perylenequinoid pigments (PQP) identified to date include hypocrellins, cercosporin, phleichrome, cladochrome, elsinochromes, erythroaphins, and calphostins. Most of them are produced by a wide variety of molds, and act as the photodynamic phytotoxins of their hosts, except that erythroaphins are isolated from aphids. For their general chemical properties [see Weiss, et al., *Prog. Chem. Org. Nat. Prod.*, 52:1 (1987) and Diwu, et al., *Photochem & Photobiol.*, 52:609-616 (1990)]. PQP's general photophysical and photochemical properties have been reviewed in Diwu, et al., *Pharmac. Ther.*, 63:1 (1994).

Preliminary data have demonstrated that some PQPs exert a much stronger photodynamic action on EMT6/ED tumor cells than Photofrin-II®. Hypocrellins, representative of photodynamic therapy applications of PQPs, exhibit several advantages over the presently used hematoporphyrin derivatives, for example, ready preparation and easy purification, high triplet quantum yield, strong red light absorptivity, and significantly reduced normal tissue photosensitivity due to their rapid excretion. Many of PQP's properties are summarized in Diwu, et al., *J. Photochem. Photobiol. A: Chem.*, 64:273 (1992). Some perylenequinones are also potent inhibitors of certain viruses, particularly human immunodeficiency virus (HIV), and also the enzyme protein kinase C (PKC). Both anti-HIV and anti-PKC activities of certain PQPs are light-dependent, a phenomenon implicated in the photodynamic therapy of cancers [Diwu, et al., *Biochem. Pharmacol.*, 47:373-389 (1994)]. The Diwu et al paper also discloses the successful conjugation of HB to a protein.

As in the case of hematoporphyrin derivatives, natural PQPs do not themselves exhibit absorptivity longer than 600 nm, a characteristic that inherently describes a decreased capability of penetrating tissues. This means that the natural PQPs are not sufficiently strong for photodynamic therapy, and this limits their photodynamic

therapy applications.

Deficiencies of current porphyrin photosensitizers for photodynamic therapy have stimulated the development of a series of second generation compounds which have improved properties with respect to light absorption in the red spectral range, purity, pharmacokinetics, and reduced cutaneous photosensitivity. The inventors have concentrated on development of derivatives of the perylenequinone, hypocrellin B. The inventors have identified compounds with excellent physicochemical and biological properties and have completed comprehensive biological characterizations and preclinical evaluation, e.g., Fig. 2, HBEA-R1 (Structure 3) and HBBA-R2 (structure 6).

These compounds, in the presence of 630 or 688 nm light, photopotentiate by a factor of 100-500, with respect to their dark cytotoxicity. HBEA-R1 (Structure 3) and HBBA-R2 (structure 6) have successfully passed dose escalation studies in rodents. No problems of acute or chronic toxicity (60 days or more) were observed following total body doses of 50 μM. Endpoints considered were body mass, behavior, and gross pathological examination at the end of the observation period. A pharmacokinetic study was completed in Balb/c mice bearing the EMT6/Ed tumor, using ^{14}C-hypocrellin B (Liu et al., 1995). The information from the HB study provided useful guidelines for timing light administration (Table 1) in the tumor growth delay/control studies with HBEA-R1.

Brief Description of the Drawings

Figure 1.: Schematic for the preparation of a variety of aminated Hypocrellin B derivatives. R can be an alkyl, alkenyl, alkynyl or an alcohol containing 2 to 10 carbon atoms.

Figure 2: Chemical structures of hypocrellin A, hypocrellin B, and hypocrellin derivatives , two butylaminated HB structures, two ethanolaminated HB structures, 2-(N,N-dimethylamino)propylamine-HB, and JL-1-1. The phenolic hydroxyl groups of the parent compounds provide a convenient site for modification.

Figure 3: Absorption spectra for HA (dimethyformamide), HB (dimethylformamide), HBBA-R2 (chloroform), HBDP-R1 (chloroform), HBEA-R1

(dimethylsulfoxide), and structure 4 (JL-1-1) (chloroform). Ordinate: Absorbance; Abscissa: Wavelength, nm.

Figure 4. Schematic for the synthesis of radiolabelled hypocrellin B. This compound can then be used to synthesize radiolabelled derivatives of hypocrellin.

Figure 5a-b : Cellular uptake of selected photosensitizers. (a). Standard concentration curves; (b). Uptake by EMT6/Ed cells, adjusted to 10^6 cells. Ordinate: Relative fluorescence at the appropriate detection wavelength (see text); *abscissa*: Concentration of photosensitizer, micromolar.

Figure: 6a-e: Survival curves of cytotoxicity (**a**) ; (*ordinate* percent survival, *abscissa* photosensitizer concentration, μM) and phototoxicity (**b-e**); (*ordinate* percentage survival, *abscissa* light dose J/ cm^2) of hypocrellin sensitizers, determined by clonogenic assays of EMT6/Ed cells in monolayer culture. **b**. HBBA-R2, **c** HBEA-R1, **d** HBDP-R1, **e** JL-1-1. Error bars represent standard deviations of five replicate culture plates.

Figure 7. Tumor growth delay under various conditions of HBEA-R1 PDT, of the EMT6/Ed tumor growing in the flanks of Balb/c mice. Transcutaneous illumination (non-hyperthermic).

Figure 8. The effect of light intensity in the for curing tumors in mice in the presence of HBEA-R1. The mice were Balb/c with EMT6/Ed tumours growing in the flanks. The mice were injected with 50 μmol/kg of HBEA-R1 prior to transcutaneous illumination.

Figure 9 a-e. Uptake of (a) hypocrellin B, (b) ^{14}C-HB, (c) HBBA-R2, (d) HBEA-R1 and (e) HBDP-R1 into EMT6/Ed cells versus time.

Figure 10. Oxygen dependency of HBEA-R1 and HBBA-R2 following graded doses of 630 nm light and 0.25 μM photosensitizer. Both compounds demonstrate phototoxicity in the absence of oxygen, providing evidence for the type I photochemical process under this condition.

Figure 11. Apoptotic cell death induced as a function of time following PDT treatment of EMT6/Ed cells in monolayer as determined using the propidium iodide method. Sensitizer concentration, 0.20 μM.

Figure 12. The effect of pH on EMT6/Ed cell cytotoxicity (a) hypocrellin A

and 630 nm light, (b) hypocrellin A in the dark, (c) hypocrellin B and 630 nm light, and (d) hypocrellin B in the dark.

Figure 13 shows the synthesis of amino acid derivatives of hypocrellin B.

Figure 14 shows the structure of additional amino acid derivatives of hypocrellin B.

Figure 15 shows a scheme for producing hypocrellin B derivatives according to the present invention.

Figure 16 shows a prior art process for producing certain precursor compounds.

Modes For Carrying Out the Invention

The present invention comprises the use of perylenequinone (PQP) derivatives as photodynamic agents, and the use of PQP derivatives in photodynamic therapy (PDT). PDT offers a unique treatment alternative for malignancies and the like that are resistant to conventional therapies, with the potential for selective destruction of malignant cells (as shown herein).

The invention also comprises a method of treating a disease by administering a therapeutically sufficient amount of at least one PQP derivative, and activating the derivative(s), typically by photoactivating the PQP derivative. Typically, the PQP derivative may be activated by exposing the derivative to a pre-determined wavelength of light. The invention also includes a method of treating cancer which is enhanced in the presence of light wavelengths between about 400 nm and about 850 nm. The absorption spectra for many compounds is shown in Figure 3, and the main absorption peak for each compound is listed in Table 1. Many of these compounds have significant absorbance in the 600 nm to the 700 nm range (see Table 1).

The invention also comprises using one or more PQP derivatives to generate singlet oxygen and a variety of toxic free radicals. Typically, compounds that are capable of generating singlet oxygen and/or toxic free radicals may be used to treat certain diseases and the like.

The invention also comprises using the hypocrellin derivatives that have anti-cancer and/or anti-viral activity, and enhancing the activity of these derivatives by

photoactivating the derivative. The invention also includes using the hypocrellin derivatives to preferentially destroy or preferentially target cancer cells.

The invention also comprises a method for producing native perylenequinones, such as hypocrellin, synthesizing perylenequinone derivatives, such as hypocrellin A and hypocrellin B, radiolabeled hypocrellin B and radiolabeled hypocrellin B derivatives. The invention also comprises compositions containing perylenequinone derivatives, hypocrellin derivatives, radiolabeled hypocrellin B and radiolabeled hypocrellin B derivatives, perylenequinone conjugates, and hypocrellin conjugates.

The invention also comprises conjugating the PQP derivatives of the present invention to one or more binding agents, such as antibodies or an antibody fragment. The invention also comprises the PQP derivative conjugated to one or more binding agents, such as an antibody or antibody fragments. The invention also comprises conjugating the PQP derivatives, e.g., hypocrellin derivatives, to DNA minor-groove-binding agents to effect phototoxicity in a cell structure, such as the cell nucleus.

As used herein, "perylenequinone derivative" or "derivative" refers to all compounds derived from native or natural perylenequinones and which can be activated by light of a pre-determined wavelength. In a preferred embodiment of the invention, the derivative is a compound derived from naturally occurring hypocrellin A or hypocrellin B, and hypocrellin-like compounds. Hypocrellin derivatives, as used herein, may be activated by light, and may be used as photodynamic agents. A derivative according to the invention may also be complexed with or include other active reagents, including but not limited to chemotherapeutic agents or alkylating agents. The structures of exemplary derivatives are shown in the Figures.

As used herein, "disease" refers to the management, diagnosis, and/or palliation of any mammalian (including human) disease, disorder, malady, or condition that can be treated by photodynamic therapy. "Disease" includes but is not limited to cancer and its metastases, such as skin cancer; growths or tumors, and their metastases; tumors and tumor cells, such as sarcomas and carcinomas, including solid tumors, blood-borne tumors, and tumors found in nasal passages, the bladder, the esophagus, or lung, including the bronchi ; viruses, including retroviruses; bacterial diseases; fungal diseases;

and dermatological conditions or disorders, such as lesions of the vulva, keloid, vitiligo, psoriasis, benign tumors, endometriosis, Barett's esophagus, *Tinea capitis*, and lichen amyloidosis.

As used herein, "administering" refers to any action that results in exposing or contacting one or more PQP derivatives with a pre-determined cell, cells, or tissue, typically mammalian. As used herein, administering may be conducted *in vivo*, *in vitro*, or *ex vivo*. For example, a composition may be administered by injection or through an endoscope. Administering also includes the direct application to cells of a composition according to the present invention. For example, during the course of surgery, tumor cells may be exposed. In accordance with an embodiment of the invention, these exposed cells (or tumors) may be exposed directly to a composition of the present invention, e.g., by washing or irrigating the surgical site and/or the cells.

As used herein, "binding agent" refers to any reagent or the like that forms a specific bond with a receptor carried on a target moiety, e.g., an antibody or antibody fragment that binds to the CA 125 antigen of ovarian cancer, or an agent that targets a certain region or structure(s) of a cell. In a preferred embodiment of the invention, the binding agent is an antibody or antibody fragment that specifically binds to cancer cells. In a more preferred embodiment of the invention, the binding agent binds an epitope of an antigen of ovarian cancer, breast cancer, or gastrointestinal cancer (e.g., the CA125 epitope of ovarian cancer, the CA 15.3 epitope of breast cancer, or the CA 19.9 epitope of gastrointestinal cancer).

The antibody or fragment may be labeled (e.g., with radioisotopes or other markers) or unlabeled, and/or in a complex; chimeric monoclonal antibodies ("C-MAb"); genetically engineered monoclonal antibodies ("G-MAb"); fragments of monoclonal antibodies (including but not limited to "$F(Ab)_2$", "F(Ab)" and "Dab"); single chains representing the reactive portion of monoclonal antibodies ("SC-MAb"); tumor-binding peptides; any of the above joined to a molecule that mediates an effector function; and mimics of any of the above. Various binding agents, antibodies, antigens, and methods for preparing, isolating, and using the antibodies are described in U.S. Patent 4,471,057 (Koprowski) and U.S. Patent 5,075,218 (Jette, et al.), both

incorporated herein by reference. Furthermore, many of these antibodies or binding agents are commercially available from Centocor, Abbott Laboratories, Commissariat à L'Energie Atomique, Hoffman-LaRoche, Inc., Sorin Biomedica, and FujiRebio.

As used herein, "photopotentiation factor" refers to the property of the compound(s) to exert light-mediated toxicity in excess of its (their) inherent dark toxicity. In a preferred embodiment of the invention, the photoactivation factor may be calculated as the ratio of the LD_{50} of cells treated without light to the LD_{50} of the cells treated with a light-activated compound (drug LD_{50} divided by drug and light LD_{50}). Where the term "LD_{50}" has been used above, the term "IC_{50}" may be substituted, to address the bioassays that concern metabolic activity rather than the endpoint of lethality, loss of reproductive capability, or clonogenic death. The relative photoactivation efficiency of a compound may also be determined using a clonogenic assay, an assay that is well known to those skilled in the art (see, for example, Example 13).

In accordance with the present invention, a desirable PQP derivative is one that is non-toxic (or of low toxicity) at high drug concentrations without light (i.e., "dark"), and is toxic at low concentrations when light of the appropriate wavelength is applied. As is recognized by those skilled in the art, the most desirable compounds are those that provide a wide range of non-toxic doses in the dark, as this characteristic provides an increased safety factor for the patient. As noted in more detail in the Examples, an *in vitro* photopotentiation factor test successfully indicates which hypocrellin congeners have the best *in vivo* tumor-killing potential. For example, HBEA-R1 and HBBA-R2 photopotentiate by a factor of 100 or more with respect to their dark cytotoxicities in the presence of 630 nm or 688 nm light (see Table 1).

As used herein, physiologically acceptable fluid refers to any fluid or additive suitable for combination with a composition containing a PQP derivative. Typically these fluids are used as a diluent or carrier. Exemplary physiologically acceptable fluids include but are not limited to preservative solutions, saline solution, an isotonic (about 0.9%) saline solution, or about a 5% albumin solution or suspension. It is intended that the present invention is not to be limited by the type of physiologically acceptable fluid

used. The composition may also include pharmaceutically acceptable carriers. Pharmaceutically accepted carriers include but are not limited to saline, sterile water, phosphate buffered saline, and the like. Other buffering agents, dispersing agents, and inert non-toxic substances suitable for delivery to a patient may be included in the compositions of the present invention. The compositions may be solutions, suspensions or any appropriate formulation suitable for administration, and are typically sterile and free of undesirable particulate matter. The compositions may be sterilized by conventional sterilization techniques.

In accordance with a method of the invention, the binding agent must be capable of binding a pre-determined binding site or receptor, and may be administered to the patient by any immunologically suitable route. For example, the binding agent may be introduced into the patient by an intravenous, subcutaneous, intraperitoneal, intrathecal, intravesical, intradermal, intramuscular, or intralymphatic routes. The composition may be in solution, tablet, aerosol, or multi-phase formulation forms. Liposomes, long-circulating liposomes, immunoliposomes, biodegradable microspheres, micelles, or the like may also be used as a carrier, vehicle, or delivery system. Furthermore, using *ex vivo* procedures well known in the art, blood or serum from the patient may be removed from the patient; optionally, it may be desirable to purify the antigen in the patient's blood; the blood or serum may then be mixed with a composition that includes a binding agent according to the invention; and the treated blood or serum is returned to the patient. The clinician may compare the anti-idiotypic and anti-isotypic responses associated with these different routes in determining the most effective route of administration. The invention should not be limited to any particular method of introducing the binding agent into the patient.

The compounds of the present invention may be produced by any method that results in a purified or substantially purified compound, or in a compound that is useful as a photodynamic agent. The compounds of the present invention may also form a composition comprising a cocktail of compounds, e.g., more than one compound. These methods are well known in the art, e.g., Liu, et al., "Synthetic studies in novel hypocrellin B derivatives," **Tetrahedron**, 49:10785 (1993); and Diwu, et al., **Anti-**

Cancer Drug Design, 8:129-143 (1993). Hypocrellin derivatives may be readily synthesized from the parent compound, hypocrellin B (HB), a natural product of the fungus *Hypocrella bambusae sacc.*, a phytopathogen of bamboo. The parent compound may also be produced synthetically as shown in the Examples. Exemplary methods of producing the compounds are also shown in more detail in the Examples. It is intended that the invention is not to be limited by the method of producing, isolating, or purifying the hypocrellin derivatives.

Briefly, for the synthesis of HA, crude HA can be prepared by acetone extraction of *Hypocrella bambusae* (B. And Br.) *Sacc.* Lipids can be removed by counter extraction with petroleum ether. Further purification can be carried out on a silica gel column, followed by 1% potassium dihydrogen phosphate-silica gel thin-layer chromatography and recrystallization from acetone.

For the synthesis of HB, crude HB can be prepared by quantitative potassium hydroxide dehydration of HA, followed by neutralization with HCl chloroform extraction and recrystallization from benzene-petroleum ether. The product was subjected to 1% citric acid-silica gel thin-layer chromatography using a 6:2:1 mixture of petroleum ether acetate ethanol as eluent. HB derivatives, HBBA-R2 (butylaminated HB), HBDP-RI (2-(N,N-dimethylamino)-propylamine-HB), and HBEA-RI (ethanolaminated HB) were prepared by amination of the phenolic hydroxyl groups of the parent compound. JL-1-1 (structure 4) was prepared according to the method of Liu, et al., *Tetrahedron* 49:10785 (1993). The absorption spectra of these derivatives were determined on a Hewlett-Packard diode array spectrophotometer (see Figure 3).

Intracellular uptake may be rapid (e.g., within 2 hours for HBEA-R1 and HBBA-R2), or may uptake may require more time (e.g., about 20 hours for HBDP-R1). Some degree of selective tumor uptake might be achieved by modification of the pKa of the sensitizer, since the interstitial milieu of some tumors is more acidic than that of normal tissues. This invention includes a method for identifying compounds where the toxicity of the compounds is higher for cancer cells than for normal cells, via comparative clonogenic assays (see Figure 12 and Example 13).

The PQP derivatives of the present invention may also be used in conjunction

with and conjugated to a number of other compounds, signaling agents, enhancers, and/or targeting agents. For example, a hypocrellin derivative of the present invention may be conjugated to an antibody, preferably a monoclonal antibody. In accordance with the present invention, the binding agent includes any DNA minor-groove targeting agent, such as lexitropsin or netropsin, preferably to enhance the targeting of the phototoxicity in the cell nucleus. Suitable enhancers include but are not limited to pKa modifiers, hypoxic cell radiosensitizers, and bioreductively activated anti-neoplastic agents, such as mitomycin C (preferably to effect or potentiate the toxicity of the compound in hypoxic cells or microorganisms). Suitable signaling agents include but are not limited to markers of apoptotic cell death or necrotic cell death, or regulatory molecules endogenous to cell cycle control or delay, preferably to potentiate the phototoxicity of the compound(s) by induction of apoptotic or necrotic cell death, or by inhibition of the repair of any form of lethal or potentially lethal damage (PLD).

As noted above, an embodiment of the invention includes binding agent-PQP conjugates (or immunoconjugates) and the therapeutic use of these conjugates. In accordance with the present invention, any method of linking a binding agent to a PQP may be used. For example, it is well known how to link an antibody or an antibody fragment to a photosensitizer. For example, Goff, et al., *British Journal of Cancer*, 74:1194-1198 (1996) discloses the production of an immunoconjugate by incubating a photosensitizer with monoclonal antibody OC125, an antibody that specifically binds to the CA125 antigen associated with most ovarian cancers. In this exemplary immunoconjugate, polyglutamic acid may be bound to a monoethylendiamine monoamide derivative, which is then covalently linked to the carbohydrate moiety at the hinge region of the monoclonal antibody away from the antigen binding sites. Other exemplary linkages are disclosed in U.S. Patent 4,722,906 and 3,959,078, both incorporated herein by reference. Briefly, these patents disclose providing a photosensitizer with a selector group, or a latent reactive group, that is the other member of a specific binding pair, e.g., a reactive group that covalently bonds to an antibody.

In accordance with the present invention, the PQP derivatives may be

functionalized, e.g., include reactive groups including but not limited to an acid, hydroxyl, an acid halide (preferably bromide), a hydrazine, a thiol, or a primary amine. The binding reagent may include reactive groups including but not limited to amino acids, such as cysteine, lysine, aspartic acid, glutamic acid and other dicarboxylic acid amino acids, and other tri- or poly-functional amino acid derivatives.

In a preferred embodiment of the invention, the PQP is an amino acid derivative of hypocrellin B, as shown in Figures 13 and 14, and described in Example 20. All R groups are a potential antibody binding site. At the present time, the most preferred immunoconjugates use hypocrellin B derivative 6 in Figure 14, and which include an acid, acid bromide, hydrazine, thiol, or primary amine antibody binding site (e.g., R group). In a preferred embodiment of the invention, any of these derivatives may be linked to a monoclonal antibody that specifically binds to ovarian cancer (e.g., the CA125 epitope), breast cancer (e.g., the CA15.3 epitope), or gastrointestinal cancer (e.g., the CA19.9 epitope). In accordance with the present invention, the most preferred immunoconjugates are hypocrellin B derivative 6 (Figure 14) bound to OC 125, B43, Ar 8.1, or PDL 10 antibodies that specifically bind to ovarian cancer; DF 3 or AR 20.5 antibodies that specifically bind to breast cancer; or B67.4, NS 1116, or AR 18.4 antibodies that specifically bind to gastrointestinal cancer. Many of these, and other, binding agents are commercially available.

As is recognized by one skilled in the art, an effective dose of the derivative or a conjugate that includes the derivative will depend in part on the severity of the disease and the status of the patient's immune system. One skilled in the art will recognize that a variety of doses may be used, and are dependent on a variety of well known factors. For example, the dose would be different for diagnosis versus treatment, or for palliation versus management. Generally, the composition will include about 0.1 μg to about 2 mg or more of binding agent per kilogram of body weight, more commonly dosages of about 200 μg per kilogram of body weight. The concentration usually will be at least about 0.5%. Any amount may be selected primarily based on fluid volume, viscosity, antigenicity, etc., in accordance with the chosen mode of administration.

Administration of the conjugate or the derivative may be more than once,

preferably three times over a prolonged period. As the compositions of this invention may be used for patients in a serious disease state, i.e., life-threatening or potentially life-threatening, excesses of the binding agent may be administered if desirable. Actual methods and protocols for administering pharmaceutical compositions, including dilution techniques for injections of the present compositions, are well known or will be apparent to one skilled in the art. Some of these methods and protocols are described in *Remington's Pharmaceutical Science*, Mack Publishing Co. (1982).

In accordance with another embodiment of the invention, a composition of the present invention may be administered alone, in combination with other compositions, or in sequence with other PDT compositions. For example, it has been disclosed above that the cellular uptake for HBEA-R1 and HBBA-R2 is rapid and distributed primarily in cytoplasmic elements. In contrast, it has also been disclosed above that HBDP-R1 reaches peak intracellular concentrations after approximately 20 hours, and is distributed primarily to cellular membranes. These features afford potential augmentation of the photodynamic therapeutic ratio through sequential sensitizer administration (followed by light treatment). Under these conditions, a larger number of organelles can be targeted.

In this embodiment of the invention, a PDT method comprises administering a first photodynamic agent, preferably having a slow uptake, and administering a second photodynamic agent, preferably having a more rapid uptake than that of the first agent. Both first and second photodynamic agents may then be activated by exposing the patient and/or the agent to light of suitable wavelength, as described above.

The excellent fluorescence properties of the hypocrellins and derivatives provide a valuable tool to monitor intracellular uptake and distribution kinetics by confocal laser scanning microscopy (CLSM). Each drug has unique properties of uptake and distribution (Figure 9) (Miller et al 1995 a,b) It is noteworthy that uptake is essentially complete within the first two hours of administration of HBEA-R1 (Structure 3) and HBBA-R2 (structure 6) (Miller et al 1995 a,b). The rate cells take up drug in humans *in vitro* and *in vivo* can be determined using similar protocols as Liu et al 1995 and Miller et al., 1995 a or b). *In vivo*, the ideal time between i.v. injection or

administration of the drug and light administration is preferably when tumor concentration of the photodynamic agent is optimal with respect to normal tissues, typically up to about 24 hours, but as long as 48 hours or more (Table 2).

Some of the embodiments of the present invention also have the added benefit of functioning with or without the presence of oxygen. Therefore, some embodiments of the present invention are effective in the treatment of solid tumors which are either well oxygenated or either partially or fully hypoxic.

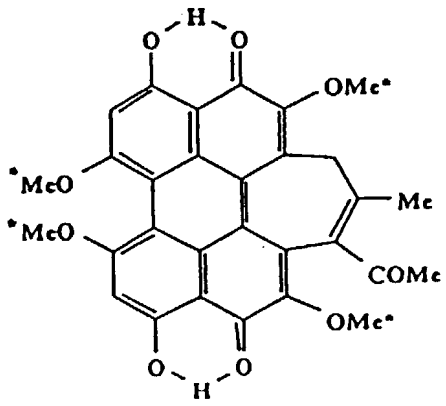
Examples

Example 1. Derivatives obtained from hypocrellin A.

Briefly, crude HA was prepared by acetone extraction of *Hypocrella bambusae* (B. *et* Br.*) Sacc.* Lipids were removed by counter extraction with petroleum ether. Further purification was carried out on a silica gel column, followed by 1% potassium dihydrogen phosphate-silica gel thin-layer chromatography and recrystallization from acetone. HB was prepared by quantitative potassium hydroxide dehydration of HA, followed by neutralization with HCl chloroform extraction, and recrystallization from benzene-petroleum ether. The product was subjected to 1% citric acid-silica gel thin-layer chromatography, using a 6:2:1 mixture of petroleum ether ethyl acetate ethanol as eluent. HB derivatives, HBBA-R2, HBDP-RI, and HBEA-RI, were prepared by amination of the phenolic hydroxyl groups of the parent compound. JL-1-1 (structure 4) was prepared according to the method of Liu et al. [1993]. The absorption spectra of these derivatives were determined on a Hewlett-Packard diode array spectrophotometer (Figure 3).

Example 2. Derivatives obtained from hypocrellin B.

The following discloses a process for synthesizing the following structure:



wherein any or all Me groups (marked with an "*") can be replaced with alkyl groups (branched or straight chain), with 2 to 6 carbons, or other chemical groups. These compounds and processes can be used to make precursors or intermediates for compounds including but not limited to anti-cancer agents, anti-viral agents, anti-retroviral agents, anti-bacteriocidal agents, anti-fungal agents, perylenequinones and derivatives, hypocrellins and derivatives, hypocrellin A and derivatives, hypocrellin B and derivatives, cercosporin and derivatives, phleichrome and derivatives, elsinochromes and derivatives, cladochromes and derivatives, erythroaphins and derivatives, calphostins and derivatives, , and other compounds with photodynamic activity.

An efficient total synthesis of HB using precursor 6 is shown in Figure 15. Commercially available 3,5-dimethoxybenzaldehyde was converted to compound 5 using seven reaction steps, according to the procedure shown in Hauser, et al, *J. Org. Chem.* 59:1967 (1994). Oxidation of compound 5 with benzeneseleninic anhydride in anhydrous THF produced the 1,2-naphthoquinone 6 in 87% yield. Attempted coupling of compound 6 to the corresponding perylenequinone by TFA and $FeCl_3$ was not successful, but coupling of its ester, which was prepared in the presence of Ac_2O,

pyridine, and a catalytic amount of DMAP in 95% yield, gave perylenequinone 7 in 88% yield. Methylation of 7 with methyl iodide and C_5F in anhydrous THF led to compound 8 quantitatively. Hydrolysis of 8 in K_2CO_3 and MeOH-H_2O solution, followed by oxidation with CrO_3 and pyridine in CH_2Cl_2 gave the methyl ketone 1 in 63% overall yield. Compound 9, which was obtained from 1 in 85% yield by an intramolecular aldol condensation reaction, was demethylated selectively with 48% hydrobromic acid to afford a 1:1 mixture of enantiomers HB (90%) which was identical in all respects (TLC, IR, NMR) with the natural product HB. This synthesis provides an entry to the synthesis of other perylenequinones.

1-acetyloxy-3-acetonyl-6,8-dimethoxynaphthalene (3): this compound was prepared using six reaction steps from 3,5-dimethoxybenzaldehyde according to the procedures disclosed in Hauser, et al, *J. Org. Chem.* 59:1967 (1994).

1-hydroxy-3-acetonyl-6,8-dimethoxynaphthalene (4): to a solution of 3 (624 mg., 2.07 mmol) in MeOH (20 ml) was added K_2CO_3 (1.0 g) in water. (10 ml), and the mixture was stirred for two hours, acidified with 5% Hcl, diluted with water (100 ml), and extracted with $CHCl_3$. The combined extracts were washed with water, then dried (Na_2SO_4), filtered and evaporated to give product 4 (505 mg, 94% yield). ^1H NMR: δ 9.10 (s, 1H, ArOH), 7.00 (d, 1H, J= 2.0 Hz, ArH), 6.68 (d, 1H, J= 2.0 Hz, ArH), 6.57 (d, 1H, J= 2.0 Hz, ArH), 6.40 (d, 1H, J= 2.0 Hz, ArH), , 4.00 (s, 3H, OMe), 3.88 (s, 3H, OMe), 3.70 (s, 2H, CH_2), 2.18 (s, #H, Me). HRMS (m/e) $C_{15}H_{16}O_4$): (M) calc. 260.10486; found 260.10472.

(±) -4-hydroxy-5,7-dimethoxy-α-methylnaphthalene-ethanol (5): this compound was prepared according to the procedures disclosed in Hauser, et al, *J. Org. Chem.* 59:1967 (1994).

(±) -3-[2-(hydroxy)propyl]-6,8-dimethoxy-1,2-naphthaquinone (6): a solution of 5 (440 mg, 1.53 mmol) in dry THF (15 ml) was added dropwise during 15 minutes to a stirred suspension of 70% benzeneseleninic anhydride (900 mg, 1.75 mmol) in THF (15 ml) at 50°C and stirring was continued for 10 minutes. The reaction mixture was

poured into water (100 ml) and extracted with $CHCl_3$. The extract was washed with aqueous $NaHCO_3$ solution (10%) and water, and then dried. Evaporation followed by column chromatography on silica gel using CH_2Cl_2:MeOH (20:1 v/v) as an eluent gave the product 6 (402 mg, 87% yield) as an orange solid, m.p. 70-72°C. ^1H NMR: δ 7.12 (s, 1H, ArH), 6.38 (d, 1H, J= 2.0 Hz, ArH), 6.36 (d, 1H, J= 2.0 Hz, ArH), 4.04 (m, 1H, CHOH), 3.91 (s, 3H, OMe), 3.89 (s, 3H, OMe), 2.67 (m, 1H, CHaHb), 2.62 (m, 1H, CH'aH'b), 2.41 (m, 1H, CHaHb), 2.36 (m, 1H, CHa'H'b), 1.25 (s, 3H, Me) 1.24 (s, 3H, Me'). HRMS (m/e) $C_{15}H_{16}O_4$): (M) calc. 276.09976; found 276.09882.

3-Acetonyl-6,8-dimethoxy-1,2-napthoquinone (2):

This compound was prepared in 81% yield from 4 in a similar manner as that described for 6. This compound is unstable and can not be purified by chromatography, but it can be recrystallized from $CHCl_3$, melting point 185°C (dec.). ^1H NMR: δ7.19 (s, 1H., ArH), 6.46 (d, 1H, J = 2.0 Hz, ArH), 6.44 (d, 1H, J = 2.0 Hz, ArH), 3.97 (s, 3H, OMe.), 3.92 (s, 3H, OMe), 3.55 (s, 2H, CH_2), 2.29 (s, 3H. Me). HRMS (m/e) $C_{15}H_{16}O_5$: (M + 2H) calc. 276.09976; found 276.09946.

To a solution of 6 (300 mg. 1.09 mmol) in $CHCl_3$ (10 ml) was added Ac_2O (1.5 ml), pyridine (1.5 ml) and 4-dimethylaminopyridine (DMAP) (100 mg). The reaction mixture was stirred for 3 hours at room temperature, diluted with H_20 (100 ml), and extracted with $CHCl_3$. The extracts were washed with 1N HCl and H_20 respectively. The residue was purified by flash chromatography on silica gel using CH_2Cl_2:MeOH (50:1, v/v) as an eluent to give the ester (330 mg, 95% yield), melting point 62-64°C. ^1H NMR: δ7.07 (s, 1H., ArH), 6.42 (d, 1H, J = 2.0 Hz, ArH), 6.40 (d, 1H, J = 2.0 Hz, ArH), 5.09(m, 1H, CHOAc), 3.96 (s, 3H, OMe.), 3.92 (s, 3H, OMe), 2.76 (m, 1H, CHaHb), 2.72 (m, 1H, CH'aH'b), 2.61 (m, 1H, CHaHb), 2.56 (m, 1H, CH'aH'b), 1.59 (s, 3H, Oac), 1.27 (s, 3H, Me), 1.25 (s, 3H, Me'). HRMS (m/e) $C_{17}H_{18}O_6$: (M) calc. 318.11035; found 318.10938.

1,12-Bis[2-(acetyloxy)propyl]-4,6,7,9-tetramethoxy-2,11-dihydroxy-3,10-perylenedione (7):

A solution of the naphthoquinone (200 mg, 0.63 mmol) in $CHCl_3$ (4.0 ml) and TFA (1.5 ml) was stirred at room temperature under N_2 for 20 minutes. A solution of anhydrous $FeCl_3$ (51 mg, 0.31 mmol) in CH_3CN (5.0 ml) was slowly added dropwise over one hour. The reaction, monitored by TLC, indicated no starting material remained. The resulting mixture was diluted with H_2O (100 ml) and extracted with $CHCl_3$. The extracts were washed with H_2O, dried (Na_2SO_4), filtered and evaporated. Chromatography of the residue on silica gel using $CHCl_3$:MeOH (50:1 v/v) as an eluent gave the product (175 mg. 88% yield). ^1H NMR: δ8.09 (s, ArH), 8.01 (s, ArH), 6.76 (m, ArH), 4.83 (m, CHOAc), 4.24–4.12 (m, OMe), 3.70 – 2.86 (m, CH_2), 1.75 (s, OAc), 1.02 (m, Me), 0.90 (s, OAc), 0.88 (s, OAc), 0.59 (m, Me). HRMS (m/e) $C_{34}H_{36}O_{12}$: (M + 2H) calc. 636.22070; found 636.22067.

1,12-Bis[2-(acetyloxy)propyl]-2,4,6,7,9,11-hexamethoxy-3,10-perylenedione (8) :

To a so1ution of 7 (250 mg, 0.39 mmol) in anhydrous THF (10 ml) was added CsF (500 mg) and methyl iodide (0.5 ml). After stirring overnight at room temperature, the reaction mixture was diluted with H20 (100 ml) and extracted with $CHCl_3$. The combined extracts were dried (Na_2SO_4), filtered and evaporated. Chromatography of the residue on silica gel using $CHCl_3$:MeOH (60:1, v/v) as an eluent gave the product (245 mg, 94% yield). ^1H NMR: δ6.77 (m, ArH), 4.73 (m, CHOAc), 4.63 (m, CHOAc), 4.19–4.08 (m, OMe), 3.43 – 2.62 (m, CH_2), 1.81 (s, OAc), 1.79 (s, OAc), 1.05 (m, Me), 0.76 (s, OAc), 0.75 (s, OAc), 0.52 (m, Me). HRMS (m/e) $C_{36}H_{38}O_{12}$: (M) calc. 662.23633; found 6662.23610.

1,12-Bis[2-(hydrox)propyl]-2,4,6,7,9,11-hexamethoxy-3,10-perylenedione:

To a solution of 8 (160 mg, 0.24 mmol) in MeOH (10 ml) was added K_2CO_3 (500 mg) in H_2O (5 ml) and the mixture was stirred overnight, acidified with 5% Hcl, diluted with H_2O (100 ml), and extracted with $CHCl_3$. The combined extracts were washed with H_2O, then dried (Na_2SO_4), filtered and evaporated to give product (134

mg, 96% yield). ^{1}H NMR: δ6.76 (m, ArH), 4.19 – 4.07 (m, OMe), 3.43 – 2.64 (m, CH + CH_2), 0.91 (m, Me), 0.68 (m, Me). HRMS (m/e) $C_{32}H_{34}O_{10}$: (M) calc. 578.21521; found 578.21388.

1,12-Bisacetonyl-2,4,6,7,9,11-hexamethoxy-3,10-perylenedione (1):

To a solution of dry pyridine (300 μl) in CH_2Cl_2 (5.0 ml) was added CrO_3 (300 mg), and then the mixture was stirred 15 minutes. 40 mg (0.069 mmol) of above compound in CH_2Cl_2 (2.0 ml) was added to the solution and stirred rapidly for 1 minute. The resulting solution was washed with H_2O, dried (Na_2SO_4), filtered and evaporated. Chromatography of the residue on silica gel using CH_2Cl_2:MeOH (50:1, v/v) as an eluent gave the product (26 mg, 66% yield), melting point 171 – 173°C. ^{1}HNMR: δ6.74 (s, 2H, 2 x ArH), 4.16 (d of AB quartet, J = 16.0 Hz, 2H, 2 x CH_{2a}), 4.07 (s, 6H, 2 X OMe), 3.95 (s, 6H, 2 x OMe), 3.50 (d of AB quartet, J = 16.0 Hz, 2H, 2 x CH_{2b}). HRMS (m/e) $C_{32}H_{30}O_{10}$: (M) calc. 574.18390; found 574.18126.

3-Acetyl-4,6,8,9,11,13-hexamethoxy-2-methyl-1H-cyclohepta[ghi]perylene-5,12-dione (9):

To a solution of 1 (20 mg, 0.035 mmol) in MeOH (3.0 ml) was added LiOH (100 mg) in H_2O (1.0 ml) at 0°C. The reaction mixture was stirred for 40 minutes at room temperature, diluted with H_2O (100 ml), acidified with 1N HCl, and extracted with $CHCl_3$. The combined extracts were dried (Na_2SO_4), filtered and evaporated. Chromatography of the residue on silica gel using $CHCl_3$:MeOH (15:1, v/v) as an eluent gave the product (16.5 mg, 85% yield), melting point 154 – 156°C. ^{1}HNMR: δ6.83 (s, 1H, ArH), 6.82 (s, 1H, ArH), 4.21 (s, 3H, OMe), 4.20 (s, 3H, OMe), 4.12 (s, 6H, 2 x OMe), 4.08 (s, 3H, OMe), 4.05 (s, 3H, OMe), 3.77 (d of AB quartet, J = 11.0 Hz, 1H, CH_{2a}), 3.26 (d of AB quartet, J = 11.0 Hz, 1H, CH_{2b}), 2.28 (s, 3H, Me), 1.99 (s, 3H, Me). HRMS (m/e) $C_{32}H_{28}O_9$: (M) calc. 556.17334; found 556.17437.

Hypocrellin B (HB):

To a solution of 9 (15 mg. 0.027 mmol) in $CHCl_3$ (2 ml) was added 1.0 ml of

48% HBr with stirring for one hour at 25°C. The mixture was poured into water (10 ml), extracted with $CHCl_3$ and dried. The residue was purified by preparative TLC using $CHCl_3$ as the developing solvent to afford a 1:1 mixture of enantiomers **HB** (13.0 mg, 90% yield) which was identical in all respects (TLC, IR, and NMR) with the natural product HB.

Another synthetic approach to the production of HB derivatives based on others' disclosed processes is shown in Figure 16. Briefly, simplification of HB by retro-aldol condensation of methyl ketone provides compound **1**. Cleavage of the two biaryl bonds of **1** in a retrosynthetic sense affords 3-acetonyl-6,8dimethyoxy-1,2-naphthoquinone (compound **2**). Takuwa, et al., *Synthesis*, 315 (1993) disclosed a one-pot photochemical reaction to prepare 3-(2-oxoalkyl)-1,2-naphthoquinone, however, the process afforded only a very low yield of the precursor **2** since the two methoxy substituents in the naphthoquinone may markedly decrease the photoreactivity. Hauser, et al, *J. Org. Chem.* 59:1967 (1994) disclosed the total synthesis of calphostin D, and provided a method to prepare precursor **3** in high overall yield. Hydrolysis of **3** followed by oxidation affords **2** in high yield. The precursor **2** is unstable and was used immediately for the next coupling reaction by $FeCl_3$ or TFA but no anticipated product was obtained.

Example 3. Direct amination of hypocrellin B.

HB (5O mg) was dissolved in ethanol (5 mL) containing the amine (1 mL), and the resulting solution was refluxed for 6-18 h depending upon the individual amine used. The mixture was poured into ice-water, neutralized with 10% hydrochloric acid, and extracted with chloroform. The chloroform layer was washed with water. and dried with anhydrous Na_2SO_4 and evaporated to afford a blue solid. The solid was first chromatographed on a 1% KH_2P0_4-silica gel column with dichloromethane-methanol (gradient ratio) as an eluent to give several constituents. Each constituent was twice rechromatographed on 1% citric acid-silica gel plate using 6:1:1 petroleum ether-ethyl acetate-ethanol as developing agent to afford the individual derivatives.

Example 4. Amination of hypocrellin B with ethanolamine.

Reaction of HB with ethanolamine according to the above procedure affords five products. Compound 8 (Structure 8 in Figure 2 also referred to as 3A or HBEA-R2) and compound 3 ((Structure 3 in Figure 2 also referred to as HBEA-R1 or 3B) (Isomer B)] (Diwu et al. 1993) were identified and characterized as follows:

Structure 8 (Figure 2) (20%): R: 3270, 1717 and 1612 cm^{-1} ; ^{1}H-NMR (in DMSO-d_6): 11.46 (s, <1H, exchangeable with D_2O, phenolic OH), 1.38 (s, <1H, exchangeable with D_20, phenolic OH), 6.83 (s, 1H, ArH), 6.78 (s, 1H, ArH), 4.09 (s, 3H, OCH_3),3H, OCH_3), 3.94 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 3.85- 3.50 (m, 4H, $2NHCH_3$), 3.40-2.92 (m, 4H, CH_2OH), 2.11 (s, 3H, $COCH_3$) and 1.72 ppm (s, 3H, CH_3). MS (FAB): 615 (M+H). Calculated for $C_{34}H_{34}N_2O_9$: 614.2264; found, 614.2270.

Structure 3 in Figure 2 [also referred to as 3B or HBEA-R1 (Isomer B)] (12%): IR: 3260, 1720 and 1613 cm^{-1}; ^{1}H-NMR (in DMSO-d_6): 12.11 (s, <1H, exchangeable with D_2O, phenolic OH), 11.99 (s, <1H, exchangeable with D_20, phenolic OH), 6.47 (s, 1H, ArH), 6.35 (s, 1H, ArH), 4.03 (s, 3H, OCH_3, 3.95 (s, 6H, 2 x OCH_3), 3.93 (s, 3H, OCH_3), 3.88-3.62 (m, 4H, $2NHCH_3$), 3.2O-2.95 (m, $2CH_2OH$, 2.15 (s, 3H, $COCH_3$) and 1.90 ppm (s, 3H, CH_3). MS (FAB): 615 (M+H). Calculated for $C_{34}H_{34}N_2O_9$: 614.2264; found; 614.2268.

Example 5. Amination of hypocrellin B with butylamine.

Synthesis of structure 6 (Figure 2 structure 6 also referred to as HBBA-R2 or 4A) (Isomer A) and structure 9 (Figure 2 structure 9 also referred to as 4B) (Diwu et al. 1993). Reaction of HB with butylamine according to i.e. above procedure afforded five products. Two of these compounds were identified as follows:

HBBA-R2 (Structure 6 in Figure 2) (21%): IR: 3280, 1702 and 1616 cm^{-1}; ^{1}H-NMR: 15.65 (s, 1H, exchangeable with D_20, phenolic OH), 14.94 (s, 1H, exchangeable with D_20, phenolic OH), 6.41 (s, 1H, ArH), 6.40 (s, 1H, ArH), 4.07 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 3.93 (d, 3H, OCH_3), 3.24 (m, 4H, $2NHCH_2$), 1.98 (s, 3H, $COCH_3$). 1.26 (s, 3H, CH_3) and 1.70- 0.85 ppm (m, 14H, $2CH_2CH_2CH_3$). MS (FAB): 639 (M+H). Calculated for $C_{38}H_{42}N_2O_7$: 638.2992; found; 638.2998.

Compound 4B or HBBA-R1(structure 9 in Figure 2) (11%): IR: 3300, 1715 and 1616 cm^{-1}. ^1H-NMR: 15.40 (s, 1H, exchangeable with D_2O, phenolic OH), 15.18 (s, 1H, exchangeable with D_2O, phenolic OH), 6.48 (s, 1H, ArH, 6.33 (s, 1H, ArH), 4.01 (s, 6H, 2 x OCH_3), 3.97 (d, 1H, CH), 3.96 (s, 6H), 2 x OCH_3), 3.54 (m, 4H, $2NHCH_2$), 3.14 (d, 1H, CH), 2.16 (s, 3H, $COCH_3$), 1.69 (s, 3H, CH_3) and 1.60-0.85 ppm (m, 14H, $2CH_2CH_2CH_2$). MS (FAB): 639 (M+H). Calculated for $C_{38}H_{42}N_2O_7$: 639.2998; found; 638.2992.

Example 6. Synthesis of JL-1-1 (structure 4 in Figure 2)

JL-1-1 was produced according to the process(es) disclosed in Liu, et al., *Tetrahedron* 49:10785 (1993).

Example 7. Synthesis of HBDP-R1 (structure 7 in Figure 2)

HBDP-R1 was produced according to the process(es) disclosed in Diwu, et al., *J. Photochem. Photobiol. B: Biol.* 18:131 (1993).

Example 8. Radiolabeled derivatives.

The invention provides a method for preparing radiolabelled HB and derivatives. The method for synthesizing 14-C-HB is described in Liu et al (1995). HB was dissolved in 6.0 mL of anhydrous benzene containing 130 mg of anhydrous aluminum chloride, and the solution was refluxed for 1 hr under nitrogen. The mixture was poured into 10% aqueous ammonium fluoride, and extracted with chloroform. The chloroform layer was washed with water, dried (Na_2SO_4) and evaporated to afford a red solid that contained compounds 2,3 and 4 as illustrated in Figure 4 (Diwu and Lown 1993b). A schematic for the synthesis of radiolabeled Hypocrellin B is illustrated in Figure 4.

The red solid was dissolved in 2.0 ml of anhydrous tetrahydrofuran (THF) containing 200 mg of cesium fluoride, and then ^{14}C-methyl iodide (1.0 mCi) in 1.0 ml of anhydrous THF was injected into the sealed flask. The mixture was stirred at room temperature for 24 hr, and stirring was continued another 12 hr at room temperature

following the addition of 0.3 ml of methyl iodide. The resulting solution was added to water and extracted with chloroform. The organic layer that contained compounds **1, 5** , and **6** illustrated in Figure 4 was concentrated to about 5.0 ml by nitrogen stream evaporation.

A 48% hydrobromic acid solution (2.0ml) was added to the chloroform solution and the mixture was stirred at room temperature for 30 minutes. The reaction mixture was poured into water, and extracted with chloroform. Evaporation of the chloroform layer gave a red solid. The solid was chromatographed on silica gel using chloroform as an eluent to afford radiolabeled HB.

This compound can then be used to synthesize radiolabeled derivatives of hypocrellin B as per the procedures described herein.

These radiolabeled compounds can be used to study drug distribution in tissues and can be utilized to develop or verify drug delivery systems.

Example 9. Other aminated products

Other aminated compounds with R= to an alkyl, alkenyl, alkynyl or an alcohol containing 2 to 10 carbons (Figure 1) can be readily synthesized based on procedures described herein. Those with ordinary skill can readily ascertain or determine the appropriate solvents, other appropriate reagents and conditions without resorting to undue experimentation.

Singlet oxygen yields were determined by the 9,10 diphenylanthracene (DPA) bleaching method [Diwu and Lown 1992]. Test sensitizers were illuminated at a predetermined wavelength and the kinetics of decrease in the DPA absorption peak of 374 nm were followed as $^1O_2$ was accepted by DPA. The physical and chemical properties of hypocrellin compounds synthesized and screened for toxicity *in vitro* are listed in Table 1. Compounds with exceptionally favorable characteristics of dark cytotoxicity and photopotentiation are boldfaced in Table 1.

The molecular weight of the hypocrellins and their derivatives averages approximately 640 Daltons (range, 528 - 780 D). For perspective, this contrasts with a larger molecular weight of approximately 1,130 - 4,520 D for the mainly ester- and

ether-linked oligomers of hematoporphyrin (2 - 8 porphyrin units) thought to comprise the bulk of Photofrin®.

The hypocrellins reported in this study have absorption bands in the red spectral region (Figure 3). Compounds with significant absorption around 630 nm are highlighted with asterisks in the Absorbance Peak column of Table 1. Absorption spectra of the parent compounds, HA and HB, and of four efficient congeners that are photosensitizers, are illustrated in Figure 3. It is useful to note that singlet oxygen yield (Table 1) is not strongly correlated with photopotentiation.

Example 10. Tumor cure

Using the Balb/c mouse/EMT6/Ed tumor model, the inventors have demonstrated tumor cures using HBEA-R1 (Structure 3) and clinically relevant transcutaneous doses of 630 nm light (100 mW fluence rate). These results are summarized in Figure 8. The conditions of light treatment did not provide a hyperthermic effect, and a light dose of 100 J/cm^2 effected 80 day cures in 9 of 10 animals tested to date. In fact, no animal exhibited any evidence of tumor re-growth, even until the time of sacrifice (100 days post-treatment). At the constant sensitizer "whole body" dose of 50 μmol/kg, reduction of the light dose provided less efficient tumor control. Light delivery approximately 2 hours following HBEA-R1 (Structure 3) administration provides good tumor control in the absence of marked cutaneous phototoxicity. Following a period of dry erythema and subsequent eschar formation, skin overlying the treated tumors returned to complete cosmetic normalcy within 30 days. It is of interest that HBEA-R1 PDT ($\geq$100 J/cm^2 630) does not evoke *any* skin response in tumor-free Balb/c mice treated with 50μmol/kg HBEA-R1 at graded intervals to 48 hours. These results provide encouragement that HBEA-R1 will defeat the major limitations of prolonged cutaneous photosensitivity exerted by Photofin®.

Due to the potential for regional hypoxia in some human tumors and the requirement for oxygen for the photodynamic effect, it is important to understand the relative contributions of type I and type II photochemical reactions to phototoxicity. Findings in EMT6/Ed cells in suspension culture with stringently controlled oxygen

concentrations in the gas phase during PDT, have revealed that the type II reaction predominates. Nevertheless, there is a component of type I phototoxicity when cells are sensitized with HBEA-R1 or HBBA-R2 (structure 6) in the absence of oxygen.

Example 11. Cellular Uptake of Photosensitizers

Standard curves of fluorescence yield vs. concentration were prepared for selected compounds dissolved in 3.6 mL dimethylsulfoxide (DMSO) mixed with unlabeled EMT6/Ed cells which had been removed from Petri dishes with 0.4 mL of 0.5% Tween 20 in 1 *N* NaOH. This procedure resulted in equivalence of the standard curves with the actual cell uptake assays. Cells labeled for two hours with hypocrellin B and selected congeners were rinsed three times with phosphate-buffered saline to completely remove excess drug, removed from Petri plates as described above, and added to DMSO. The washing procedure was monitored spectrophotometrically. The Tween 20-NaOH solution used to remove the cells from the plates resulted in complete cytolysis and extraction of the hypocrellins from the cells. Fluorescence was quantified with a Spex Fluoro Max™ fluorometer. The illumination and detection slit widths were set at 1 nm. The optimal excitation wavelengths were determined for each compound. For example HB excitation was 438 nm, HBBA-R1, 411 nm; HBEA-R1, 352 nm; HBDP-R1, 410 nm; and JL-1-1, 594 nm. Uptake per 10^6 cells was determined by regression analysis of the individual uptake curves, and is outlined below. A standard, 2-hour incubation interval was chosen to match the empirically chosen cytotoxicity/phototoxicity pre-incubation. Intracellular uptake kinetics experiments performed by fluorescence confocal microscopy demonstrate that intracellular uptake is complete within the two hour incubation interval (Figure 9) (Miller, et al. 1995 a,b).

Example 12. Cell Line and Culture Conditions

EMT6/Ed mouse tumor cells have been propagated by the inventors for several years [Chapman et al. 1983]. They are maintained as monolayer cultures in Waymouth's medium containing 12.5% fetal calf serum, at 37°C in a humidified

atmosphere of 95% air and 5% CO_2. They require twice weekly transfers to maintain their exponential growth. A minimum of three times per year, the cell line is passaged as solid tumors in BALB/c mice. Newly passaged EMT6/Ed cell lines are reestablished and propagated for at least two weeks *in vitro* prior to experimentation. This procedure maintains the malignant phenotype.

Example 13. Photosensitizers and Clonogenic Assays

Purified hypocrellin derivatives were obtained in lyophilized form. One day prior to each experiment, exponentially growing EMT6/Ed cells (25,000/mL in 2.0 mL) were seeded in 3.0 cm tissue culture Petri plates. The photosensitizers were kept in lyophilized form until the day of experiment, at which time they were dissolved in DMSO. Stock sensitizer-DMSO solutions were diluted with Hank's Balanced Salts Solution and added to the cells in Waymouth's medium containing 12.5% fetal calf serum for the sensitization studies. The maximum concentration of DMSO in the incubation medium was 1% (v/v), and any effects of DMSO on cloning efficiency were controlled by series of dishes containing the appropriate concentration of DMSO, but no sensitizer. Preliminary studies indicate that the presence of fetal calf serum did not significantly influence toxicity. EMT6/Ed cells were exposed to graded doses of the test compound for 2 h, after which time the test compound was removed by repeated washing with Hank's Balanced Salts Solution. The cells were then illuminated as monolayers with graded doses of 630 nm light in room air (*vide infra*). The cells were trypsinized, counted, and plated at known density in Waymouth's media. The cultures were incubated for 6 days and subjected to standard clonogenic assay [Puck and Marcus, 1956].

The dark toxicity characteristic of each compound was assessed separately following a similar procedure for cell exposure to graded doses of the photosensitizer for 2 h. Precautions were taken to avoid exposure of the cells to light throughout the period that cells were exposed to, or contained, photosensitizer. Survival curves represent a minimum of three independent experiments. Data were corrected for plating efficiency and the phototoxicity curves were corrected for dark toxicity for each

drug concentration, where necessary.

Example 14. Illumination of the Cell Cultures

A 20 Watt argon tunable dye laser system (Coherent, Innova-20, Model CR599) with Kiton Red dye (Exciton, Dayton, Ohio) was used to generate light at 630nm. The wavelength was verified with a monochromator prior to experimentation. The light beam was transported by a single, 0.4 mm quartz optical fibre and oriented 9.3 cm. above the cell culture dish. The terminus of the fibre was fitted with a lens which evenly illuminated the flat surface of the irradiation table. Rotation of the table during illumination optimized uniformity of light distribution. The dose at the surface of the cell culture was determined by a power meter (Coherent - 210) to be 1 J/ cm^2. Illuminations were performed at ambient temperature (~23°C) and the longest exposure time was 1 min.

The sensitizer uptake studies were performed under incubation conditions identical to those used for clonogenic assays. Figures 5 a. and b. demonstrate the standard curves and cellular uptake data, respectively. The molar quantity of sensitizer required to effect 50% lethality per 10^6 cells in the dark was estimated by inspection of the dark toxicity survival curves Figure 6 a., (*vide infra*) and observation of intracellular concentration achieved for the appropriate extracellular drug concentration from Figure 5b. For HB, this value is 8 nanomoles; HBEA-R1, 120 nanomoles; and JL-1-1, 32 nanomoles. Application of 630 nm light markedly reduced intracellular photosensitizer quantities required to achieve 50% cell killing. For light doses up to J/ cm^2, 160 pmole/10^6 cells of HB, or a 50-fold reduction in bound drug is required. HBEA-R1 is effective at 1 nmole/10^6 cells (120-fold less); HBDP-R1, 11 nmole/10^6 cells; and JL-1-1, 0.32 nmole/10^6 cells, a 100-fold photopotentiation of 50% lethality. Among the compounds tested, the concentration range for LD_{50} in the clonogenic assay ranged from approximately 10 μM to >100 μM (mean, approx. 25 μM). In the presence of 630 nm light, the LD_{50} photosensitizing dose of hypocrellins was reduced (0.15 to >6 μM mean, approx. 3 μM), in some cases, substantially. HBBA-R2 demonstrated up to 500 - fold photopotentiation *in vitro*, while HBEA-R1, HBDP-R1 (Figure 2 structure 7)

and JL-1-1 were characterized by 167-, 50-, and 35-fold photopotentiation factors, respectively. The photopotentiation values based on clonogenic assays generally vary proportionately with drug uptake, for those compounds for which both values were determined.

Survival curves of the *in vitro* dark toxicity of four hypocrellin derivatives are displayed in Figure 6a. Error bars represent the standard deviations of 5 replicate culture plates from three independent experiments for each compound. Each derivative has a characteristic cytotoxicity. HBBA-R2 and JL-1-1 did not evoke 50% lethality within the concentration range tested ($\leq$80 uM). HBDP-R1 produced 50% cell death at concentrations in excess of 40 μM. HBEA-R1 is effective in the 20 μM range. Many of the compounds in table 1 are photopotentiated and the LD_{50} for many of these compounds are lower than HA or HB.

Some cancer cells are slightly more acidic than normal cells therefore compounds that are more active at a pH of 6.5 than 7.0 are desirable for treating some cancers. HB (Figure 12c and d) and some of the other derivatives are most active at pH 6.5 ($\pm$ 0.5) and should be more active in acidic cells. The ability of HA to kill EMT6/Ed cells, on the other hand, does not appear to be affected by changes in pH from 5.5 to 7.5 (Figure 12a and b).

The excellent photopotentiation characteristic of HBBA-R2 is due to extremely low dark cytotoxicity on the one hand, and excellent photo-sensitization, on the other (Figures. 6a and b.). HBEA-R1 is another excellent photosensitizer, with a reciprocal drug-light dose response in the 0.15-0.3 μM, 0.25 - 1.00 J/ cm^2 ranges (Figures 6a and c.). Data presented in Figure 6d. indicate that the LD_{50} or phototoxicity of HBDP-R1 lies in the 0.5- 2.5 ,μM range, in the presence of 0.75 J/ cm^2, 630 nm light. Finally, the toxicity of JL-1-1 are represented in Figure 6e. Again, there is a reciprocal relationship between drug and light dose, with excellent phototoxicity in the 2.0 - 4.0 μM drug concentration range.

The activity of HBEA-R1 and HBBA-R2 activity is effected by oxygen levels (Figure 10). Both of these compounds are able to kill cells in the absence of oxygen or at low oxygen levels. At the low oxygen levels higher doses of light are required to kill

cells than at higher oxygen levels. Since these compounds can kill cells at low oxygen levels these compounds are suitable for the treatment of solid tumors, wherein hypoxia may be present.

Example 15. Site-specific Modification of parent structures

The basic structures of the parent hypocrellins render them amenable to site-specific modification [Diwu and Lown 1990]. A major advantage of hypocrellins as photosensitizers for photodynamic therapy, rests in their ability to be synthesized in pure, monomeric form. This feature significantly facilitates studies on phototoxic mechanisms *in vitro*; however a major advantage is foreseen in the simplicity with which pharmacokinetic studies may be executed. The low molecular weight facilitates rapid distribution among tissues. The octanol-water partition coefficient is amenable to site- specific alteration, a feature which will expedite synthesis of a series of derivatives of graded hydrophobicity. For example, addition of the terminal hydroxyls and the quaternary nitrogen atoms in the sidechains of HBEA-R1 and HBDP-R1, respectively, may promote water solubility. This property will affect association with plasma proteins and lipoproteins, and therefore, the tumor and normal tissue distribution and kinetics of uptake and clearance. Butylamino- substitution of the phenolic hydroxyls, yielding HBBA-R2 and HBBA-R1 has raised the extinction coefficient (ϵ) over six-fold compared with HB, with a concomitant 7-10-fold enhanced phototoxicity. The same substitution resulted in at least a 5-fold reduction of cytotoxicity, compared with the parent compound. The ethanolamine substitution resulting in HBEA-R1 has enhanced ϵ to a similar degree, with attendant heightened phototoxicity. Finally, 2-N,Ndimethylamino)propyl- amination of HB's phenolic hydroxyl (HBDP-R1) resulted in a 4.8-fold boost in ϵ, with augmented phototoxic activity. While these three modifications actually reduced singlet oxygen yield to varying degrees, enhanced photoxicity may relate to altered intracellular sensitizer distribution, with advantageous targeting of the phototoxic species. Of more than 25 hypocrellin derivatives screened to date, all have shown acceptable levels of cytotoxicity *in vitro*. At least seven of the compounds photopotentiate by a factor of 10 (Table 1), with four possessing

exceptional photopotentiating ability. Direct molar comparison of the hypocrellin concentrations required to effect a given degree of cytotoxicity or photopotentiation, with those for P-II® is impractical, since the molecular weight of the photosensitizing component(s) of P-II® is not clearly defined. Considering the low molecular weights characteristic of the hypocrellin derivatives, and their predominance as monomeric forms, the inventors did not deem it necessary to adhere to the tradition of longer drug pre-incubation times characteristic of some porphyrins. Pre-incubation kinetics studies confirm that varying the pre-incubation time for intervals up to 24 hours has no significant effect on the drug concentration required to exert 50% cytotoxicity or photopotentiation of cell kill. Recent fluorescence confocal microscopy studies indicate that for most compounds examined, uptake is complete within two-hour incubation period. Kinetics studies are essential to avoid premature rejection of potential photosensitizing compounds.

These *in vitro* clonogenic screening assays provide a method to screen for compounds with better physicochemical properties (and ideally, cytotoxicity and photopotentiation) of promising hypocrellin derivatives. This approach has been useful and efficient to estimate sensitizer concentration ranges required to affect cytotoxicity and photopotentiation. This method can be used to select compounds suitable for preclinical and clinical evaluations.

Other mechanistic studies concern the effect of photosensitizer and PDT on the cell cycle. Preliminary findings for HBEA-R1 and HBBA-R2 gave no indication of major cell cycle perturbations in the presence or absence of light treatment. This finding is important, since treatment induced synchrony or selective toxicity could result in residual PDT-resistant tumor cell populations.

The inventors have also found evidence that both of these sensitizers promote substantial apoptotic cell death following PDT. Control and treatment of EMT6/Ed monolayer cells were subjected to propidium iodide staining and zero-integrated field electrophoresis (ZIFE). Both techniques provided data implicating apoptosis as a major contributor to PDT toxicity (Figure 11).

Example 16. *In Vivo* Studies

The inventors have performed *in vivo* studies in rodents to assess putative systemic toxicity and pharmacokinetic properties of selected compounds. These compounds are lipophilic and have a tendency to aggregate in aqueous solutions. The preferred routes of administration of drug not in a liposome formulation is either via injection into or very near the tumor or intravenously (i.v).. After i.v. injection of HB into Balb/c EMT6/Ed cells, the maximum drug concentration in the tumor was highest between the time of injection and 2 hours after injection (Table 2). This timing of cellular uptake agrees with the *in vitro* data (Figure 9a.). The level of HB in the skin initially dropped quickly immediately after administration of the drug and the majority of the drug is cleared from the body within 24 hours(Table 2).

Preliminary analysis of HBEA-R1 and HBBA-R2 indicates that these two compounds retain significant potency *in vitro* at 688 nm. Graded doses of HBEA-R1 and HBBA-R2 have been administered to Balb/c mice, to a maximum of 31 and 39 mg/kg body weight, (50 μM) respectively. Acute and chronic toxicity studies in rats with HBEA-R1 and HBBA-R2 demonstrate no significant effects to graded doses including 7.5 mg/kg total body weight 11-12 μM. No problems of acute or chronic toxicity (60 days) were observed following total doses of 50 μM of these drugs. Primary endpoints included behavioral and physiological signs, such as consitutive grooming, hunching, altered gait, lethargy, aggression, and breathing rate and body mass compared with control animals.

Example 17. Laser Light Wavelength and Dosage

Both the concentration of drug and the dosage of light are important for treatment of tumors. Balb/c mice with EMT6/Ed tumors with 50 μmol/kg body weight of HBEA-R1 received various light dosages. The mice that received 100 Joules of 630 nm light (duration approximately 10 minutes) experienced approximately 90% tumor cure, mice that received 50 Joules of 630 nm of light experienced only a 40% cure rate and the cure rates were significantly lower at the lower light dosages (Figures 7 and 8).

This invention provides a method for treating cancer which is enhanced in the presence of light wavelengths between 400 and 850 nm (see Figure 3 and Table 1 or optimal wavelenghts for individual compounds). The absorption spectra for many compounds are included in Figure 3 and the main absorption peak for each compounds are included in Table 1. Many of these compounds have significant absorbance around the 630 nm (600 to 700 nm range) (Table 1). The optimal wavelength is different for each compound (Table 1). For HBEA-R1 and HBBA-R2 wavelengths between at least 630 and 688 nm are capable of killing cells. For deeper or larger tumors the longer wavelengths are preferred. For superficial tumors, laser wavelengths with lower wavelengths or wavelengths in the green spectrum would be more suitable to use (Nseyo et al., 1993) since the light does not penetrate as far. The ability of these compounds to be photopotentiate at higher wavelengths increases the size of tissue that can be treated with PDT and increases the depth at which treatment can be provided using PDT. Fibre optic probes can be utilized to direct the laser light. Light may also be delivered to a selected area, using an appropriate light source and shielding.

A method for treating bladder is described by Nseyo and associates (1993) this method can be applied using the compounds described in Table 1 or Figure 2 and drug doses described and wavelengths described herein.

For applications of drug to a localized region or with identifiable target antigens there are several methods that are suitable for delivery, the delivery system are comprised of drug- liposome formulations, drug -monoclonal antibody delivery systems such as monoclonal antibody -liposome, or applied to exposed surfaces using a standard lipophilic skin cream. The drug can be applied topically or the route of delivery of the drug or drug and delivery system could be intravenous, intraperitoneally, intrathecally, intravesically, by intratumoral injection or by oral ingestion.

Example 18. Composition Delivery

These compounds can be delivered to cells via liposome-mediated drug delivery, including conventional and "Stealth" liposomes, and immunoliposomes designed to

target the specific organ or cancer type. For example, liposomes containing one of the compounds described in Figure 2 with a monoclonal antibody (mAb) 48-127 (from the laboratory of Y. Fradet) attached to the surface could be used to target bladder cancers (de Harven et al. 1992). 48-127 is a mAb which recognizes a surface glycoprotein found on both human bladder transitional cell carcinoma (TCC) and AY-27 rat TCC.) (Fradet et al. 1992, 1986; Fradet and Cordon-Cardo, 1993; Rao et al. 1993). An example of how to prepare a stealth immunoliposome is as follows: liposomes containing the particular drug can be prepared from hydrogenated soy phosphatidylcholine and cholesterol and 5% PEG-DSPE (polyethylene glycol-diestearoyl phosphatidylethanolamine) using the protocol described by Allen and associates (1991). An antibody can then be covalently attached to the PEG terminus using a thioester bond as detailed by Allen (1994). Many other methods for preparing liposomes and immunoliposomes are described in the literature and patents and would be suitable carriers for these drugs. Liposomes and immunoliposomes can be made containing one or more of the compounds described herein using published procedures. Those with ordinary skill can readily ascertain or determine the appropriate solvents, other appropriate reagents and conditions without resorting to undue experimentation.

Example 19. Method for Identifying and testing new PDT photosensitizers

The desired characteristics for a PDT sensitizer comprise at least one or more of the following characteristics: good absorption of light in a wavelength that penetrates tissue to the desired depth (Absorption in the 600 nm to 850 nm range penetrate the skin many mm), compound sensitive to pH - inactive, lower activity or activity destroyed at the pH characteristic of normal tissues, but active or higher activity at the pH of the cells or organisms to be treated; compound cleared from the body quickly and if a compound is intended to treat solid tumors it should have the ability to function either in the presence and/or absence of oxygen to address the problem of tumor cell hypoxia. The photosensitizer should have low dark cytotoxicity, and excellent photopotentiation of cellular damage. The PDT toxic effect may be mediated via necrotic, apoptotic cell death, or by stasis of the tumor vasculature or vascular bed.

Example 20. Synthesis of Amino Acid Derivatives of Hypocrellin B for Conjugation Purposes

a. To a cooled solution (ice-bath) of 1 (7.5g, 100 mmol) in 25 ml of aqueous 4 M NaOH solution was added slowly, and alternately, benzyl chloroformate (17g, 100 mmol) and 25 ml of aqueous 4 M NaOH solution (each in five portions), over a period of 30 minutes. The resulting mixture was acidified with concentrated HCl, the precipitated product was filtered and dried. Recrystallization from chloroform gave the pure product (2) in 72% yield (15.1 g).

b. A solution of 2 (6.3 g, 30 mmol) in dichloromethane (100 ml) is cooled to -5°C (ice-salt bath), and concentrated H_2SO_4 (0.3 ml) is added. The solution is saturated with isobutene, and then stirred at room temperature for 48 hours. Excess isobutene was removed at the water pump and the organic layer was washed with water (2x, 100 ml), 5% aqueous sodium bicarbonate solution (2x, 100ml), water (50 ml), and dried (Na_2SO_4). Removal of the solvent *in vacuo* produced 3 as an oil, in 85% yield (6.8 g).

c. Hydrogenation of 3 (7.95 g, 30 mmol) in 80 ml of absolute ethanol in the presence of 2.0 g of 5% Pd-C at room temperature and pressure was accomplished in 3 hours. After removal of the catalyst, the resulting solution was concentrated *in vacuo* to about 30 ml, and phosphorus acid (2.5 g, 30 mmol) in 60 ml ethyl acetate was added. The organic layer was removed and evaporated to give unreacted 3 (0.4 g, 5%). The aqueous layer was made alkaline with NaOH (1.4 g), and then extracted with ethyl acetate (3x, 50 ml), dried (Na_2SO_4) and evaporated *in vacuo* to produce 4 in 75% yield (2.97 g).

d. A mixture of hypocrellin B (40 mg, 0.08 mmol) and 4 (50 mg, 0.4 mmol) was refluxed in acetonitrile for 24 hours. Removal of the solvent *in vacuo* and purification by silica gel column chromatography using chloroform:methanol

(60:1, v/v) as eluent produced product 5 in 70% yield (35 mg). IR, NMR, and FAB-MS indicate that the product is a mono-adduct.

e. To a solution of 5 (50 mg, 0.8 mmol) in dichloromethane (30 ml) is added slowly, trifluoroacetic acid (16 mg, 0.01 ml, 0.11 mmol) , and the mixture was stirred at room temperature for 1 hour. The mixture was then extracted with dichloromethane (2x, 30 ml), washed with water, dried (Na_2SO_4) and removal of the solvent *in vacuo* produces 6. Purification by silica gel column chromatography using chloroform:methanol (30:1, v/v) as eluent produced product 6 in 80% yield (46 mg).

f. A mixture of 6 (50 mg, 0.083 mmol) and thionyl bromide (17.3 mg, 0.083 mmol) in 15 ml of dichloromethane is stirred at room temperature for 1.5 hours, and then the organic layer is separated, dried (Na_2SO_4) and removal of the solvent *in vacuo* produces 7. Purification by silica gel column chromatography using chloroform:methanol (50:1, v/v) as eluent produced pure product 7 in 75% yield (41 mg).

Example 21. pH dependency

The effects of the pH of the extracellular milieu were tested with respect to phototoxicity to determine whether the low pH characteristic of some tumors would enhance the phototoxic effect via enhanced sensitizer uptake. EMT6/Ed cells growing in monolayer culture were subjected to PDT as outlined above, with the exception that the pH of the culture medium was adjusted incrementally in the range of 7.5 — 5.5 with exogenously supplied lactic acid (0.0 - 4.0 mM) during the 2-hour photosensitizer incubation. Survival relative to untreated controls was determined by clonogenic assay.

The effect of altering the extracellular pH on phototoxicity in EMT6/Ed cells in monolayer culture is summarized for HB between 0.0 and 5.0 μM in Figure 12. In the absence of HB, manipulation of extracellular pH with lactic acid has negligible effect on cloning efficiency. The pH effect is most pronounced at an extracellular pH of 6.5 for all HB concentrations, with the maximal effect at 3.0 μM of the sensitizer in the presence of light. Under these conditions, cytotoxicity is potentiated by a factor of 4 to

5 with respect to that at physiological pH.

Example 22. Genotoxicity

Preliminary studies were conducted on HBBA-R2 to estimate mutagenic potential. The *Salmonella typhimurium* histidine reversion system was used to detect frame shift mutations (strain TA98) or base substitutions (strain TA100) in DNA, according to Organization for Economic Cooperation and Development (OECD) Guidelines 471 and 472. The plate incorporation assay procedure (Ames assay) was used, with HBBA-R2 introduced into the culture mixture to a maximum of 1.0 mg/plate in 100 μl of dimethyl sulfoxide (DMSO). This sensitizer concentration showed detectable cytotoxicity in both test strains.

Table 3 below summarizes results of experiments designed to identify and characterize putative genotoxic properties of the photosensitizer HBBA-R2. Data are indicative of properties of HBBA-R2 incubated with two strains of *Salmonella typhimurium*, TA98 and TA100, in the dark. These strains were chosen for their propensity to detect a wide range of compounds with mutagenic potential. The negative control for both strains was DMSO (solvent) applied in the same concentration as that used for the test compounds. No mutagenic activity was observed for DMSO. Positive controls included 2-nitrofluorene, sodium azide, and 2-aminofluorene. They demonstrated the expected pattern of mutagenic activity in the presence and absence of mammalian metabolic activation system (S9) in each strain. HBBA-R2 did not demonstrate any mutagenic activity in either strain, at concentrations to and including 1000 μg/plate, indicating that at clinically relevant concentrations, HBBA-R2 promotes neither base substitution, nor frame -shift mutations.

The absence of genotoxicity in these mutagenicity studies may be related to the finding that the free drugs are not found in the cell nucleus, within the detection limits of CLSM, or that they are not inherently genotoxic. Conjugates that enter the nucleus, e.g., PQPs conjugated to DNA minor groove binding agents, were not tested. Nuclear labeling may provide augmented phototoxicity through DNA targeting, with no added burden of cytotoxicity.

Table 1. Physical and Chemical Properties for Hypocrellins of Use in PDT

Name of Compound (structure no. in Figure 2)		Chemical Formula	F.W.	Abs. Peak in red Spectral region (nm)	A_{max}/solvent	A_{630}	Extinction Coefficient ($\times 10^{-5}$) (630 nm)	$^1O_2$ Yield	LD_{50} Dark (μM)	LD_{50} Light (μM)	Photo-potentiation Factor
HA (1)	Hypocrellin A	$C_{30}H_{26}O_{10}$	546	658*	0.093/DMF	0.086	0.86	0.84	15	3-5	3-5
HB (5)	Hypocrellin B	$C_{30}H_{24}O_9$	528	658*	0.118/DMF	0.100	1.00	0.74	20	1.5-2	10-13
HA-Mg^{++}	HA-$Mg^{++}(Ac)_2$	$C_{34}H_{28}O_{12}Mg$	652	616	0.958/EtOH	0.447	4.47	0.71	>25	>5	ND
HB-Mg^{++}	HB-Mg^{++}	$C_{34}H_{28}O_{11}Mg$	634	622	0.604/EtOH	0.527	5.27	0.53	10	1	10
DAHA (2)	Deacetylated-HA	$C_{32}H_{24}O_{10}Mg$	592	622	0.651/EtOH	0.570	5.70	0.51	>25	>5	ND
HBAC-R1	Cystamine-HB	$C_{32}H_{27}O_8Mg$	585	646	0.417/$CHCl_3$	0.388	3.88	0.40	12.5	1	12.5
HBAC-R2	Cystamine-HB	$C_{32}H_{27}O_8Mg$	585	600	0.337/DMSO	0.244	2.44	0.31	12.5	5	2.5
HBBA-R2 (6)	*n*-butylaminated HB	$C_{36}H_{40}N_4O_7$	780	616*	0.628/$CHCl_3$	0.619	6.19	0.32	>100	0.2-0.6	167-500
HBAM-R1	2-morpholino-ethyl-aminated-HB	$C_{42}H_{48}N_4O_9$	752	658				0.70	>25	4	>6.25
HBDD-R2	2-(N,N-diethyl-amino) ethylamine-HB	$C_{42}H_{52}N_4O_7$	696	646*	0.508/$CHCl_3$	0.055	0.55	0.36	>25	7.5	>3.3
HBDP-R1 (7)	2-(N,N-dimethyl-amino) propylamine-HB	$C_{40}H_{48}N_4O_7$	724	640*	0.463/$CHCl_3$	0.480	4.80	0.42	>25	0.5-1.5	>16.6-50

Name of Compound (structure no. in Figure 2)		Chemical Formula	F.W.	Abs. Peak in red Spectral region (nm)	A_{max}/solvent	A_{630}	Extinction Coefficient (x 10^4) (630 nm)	$^1O_2$ Yield	LD_{50} Dark (μM)	LD_{50} Light (μM)	Photo-potentiation Factor
HBEA-R1 (3)	Ethanolamine-HB	$C_{34}H_{34}N_2O_2$	614	696*	0.625/DMSO	0.623	6.23	0.60	15-25	0.15	100-167
HBEA-R2 (8)	Ethanolamine-HB	$C_{34}H_{34}N_2O_2$	614	634*	0.162/DMSO	0.127	1.27	0.70	25	7.5	3.3
HBED-R2	Ethylenediamine-HB	$C_{34}H_{36}N_4O_4$	696	614*	1.449/DMSO	1.239	12.39	0.50	>25	3-5	5-8.3
HBMA-IV	Methylamine-HB	$C_{32}H_{32}N_2O_4$	696	640	0.246/$CHCl_3$	0.246	2.46	0.80	8.5	1	8.5
DBHB	5,8-dibromo-HB	$C_{30}H_{32}O_2Br_2$	531	ND	ND	ND	ND	0.62	10	3	3.3
DMHB	demethylated HB	$C_{28}H_{34}O_2$	686	648*	0.469/EtOH	4.77	4.77	0.42	>25	3-5	>5-8.3
JL-1-1 (4)		$C_{30}H_{34}O_{12}$	578	594	0.478/$CHCl_3$	0.062	0.62	0.72	>70	2-4	>18.5

HBBA-R2, HBDP-R1, HBEA-R1, and JL-1-1 demonstrate average or lower than average toxicity, with excellent potentiation. For the purposes of this study, the LD_{50} light dose was not fixed. For the compounds tested, this dose is 0.75 - 1.0 J/cm2 of 630 nm light.

ND = not done. * Significant light absorption at 630 nm. F.W. = formula weight.

Table 2.

Tissue Uptake of ^{14}C-Hypocrellin B (dpm/g)

Tissue	0 Hours	2 Hours	24 Hours	48 Hours
Heart	113,920 ± 3,365	5,135 ± 910	7,835 ± 1,810	2,325 ± 245
Lung	651,100± 42,668	8,580 ± 655	3870 ± 525	2,975 ±360
Fat	20,550 ±715	38,570 ± 5,610	19,550 ±2,210	19,335 ± 2,335
Liver	394,190 ± 7,540	24,620 ±4,885	22,495 ± 4,440	9,215 ± 720
Spleen	151,870 ± 9,395	58,900 ± 4,205	14,970 ± 3,215	26,700 ± 11,105
Stomach	28,280 ± 145	21,630 ± 3,345	34,385 ± 8,795	12,460 ± 975
Pancreas	32,010 ± 2,165	13,185 ± 12,055	32,390 ± 11,840	16,915 ± 3,845
Ileum	45,400 ± 3,600	20,280 ± 2,850	5,800 ± 645	2,840 ± 595
Kidney	67,344 ± 950	20,855 ± 3,955	12,050 ± 1,845	4,535 ± 765
Skin	14,970 ± 74	3,130 ± 221	2,700 ± 170	1,590 ± 250
Bone	19,825 ± 2,300	3,955 ± 2,070	660 ± 215	1,125 ± 310
Brain	17,560 ± 560	3,855 ± 170	2,840 ± 275	845 ± 90
Muscle	13,665 ± 600	4,050 ± 940	2,875 ±560	1,015 ± 205
Tumor	7,885 ± 270	3,775 ± 400	2,950 ± 80	2,165 ± 470
Serum	69,975 ± 1,925	1,655 ± 170	1,020 ± 160	700 ± 240

Table 3

Mutagenicity of HBBA — R2 in Strains TA98 and TA100 of Salmonella typhimurium

Control/Test Compound	Concentration of Compounds, μg/ plate	Number of Histidine Revertants per Plate			
		TA98		TA100	
		-S9	+S9	-S9	+S9
Negative control: 100 μl DMSO/ plate	0	18 ± 1	27 ± 2	133 ± 4	157 ± 5
HBBA — R2	8	47 ± 13	41 ± 1	119 ± 9	117 ± 13
	40	29 ± 5	52 ± 4	124 ± 6	140 ±13
	200	25 ± 2	29 ± 1	160 ± 3	148 ± 10
	1000	16 ± 2	13 ± 2	125 ± 16	132 ± 12
Positive Controls					
2-nitrofluorene	5.0	1197 ± 53	-	-	-
Sodium azide	1.5	-	-	2017 ± 36	-
2-aminofluorene	10.0	-	2951 ± 390	-	2601 ± 321
± S9: Presence or absence of mammalian metabolic activation system					

While the invention has been described in some detail by way of illustration and example, it should be understood that the invention is susceptible to various modifications and alternative forms, and is not restricted to the specific embodiments set forth. It should be understood that these specific embodiments are not intended to limit the invention but, on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the invention.

Claims

What is claimed is:

1. A method of treatment comprising administering a conjugate comprising a perylenequinone bound to a binding agent, and activating the perylenequinonoid by exposing the perylenequinone to light of a predetermined wavelength.

2. The method of claim 1 wherein the perylenequinone is selected from the group comprising hypocrellins, cercosporins, phleichromes, elsinochromes, cladochromes, erythroaphins, and calphostins.

3. The method of claim 2 wherein the perylenequinone is functionalized.

4. The method of claim 1 wherein the perylenequinone is a hypocrellin.

5. The method of claim 1 wherein the perylenequinone is non-toxic at high concentrations in its non-activated state and toxic at low concentrations in its activated state.

6. The method of claim 4 wherein the hypocrellin is a derivative of hypocrellin A or hypocrellin B.

7. The method of claim 6 wherein the hypocrellin B is functionalized.

8. The method of claim 7 wherein the functionalized hypocrellin B is conjugated to a binding agent.

9. The method of claim 8 wherein the hypocrellin B is non-toxic at high concentrations in its non-activated state and toxic at low concentrations in its activated state.

10. The method of claim 1 wherein the binding agent is selected from the group consisting of an antibody, a monoclonal antibody, or a fragment thereof.

11. The method of claim 10 wherein the binding agent is an antibody that binds to an antigen of a cancer cell.

12. The method of claim 11 wherein the cancer cell is selected from the group consisting of ovarian cancer, breast cancer, and gastrointestinal cancer.

13. The method of claim 1 wherein administering a conjugate comprises administering a conjugate comprising a hypocrellin B derivative bound to a monoclonal antibody or a portion thereof.

14. The method of claim 1 wherein the binding agent binds to a DNA minor groove.

15. A method of treatment comprising administering a conjugate comprising a hypocrellin B derivative bound to a monoclonal antibody or antibody fragment, and activating the hypocrellin B derivative by exposing the hypocrellin B derivative to light of a predetermined wavelength.

16. The method of claim 15 wherein the monoclonal antibody or antibody fragment binds an epitope on a cancer cell, said cancer cell is selected from the group consisting of ovarian cancer, breast cancer, and gastrointestinal cancer.

17. The method of claim 15 wherein the hypocrellin derivative is non-toxic at high concentrations in its non-activated state and toxic at low concentrations in its activated state.

18. A composition comprising a perylenequinone conjugated to a binding agent.

19. The composition of claim 18 wherein the perylenequinone is selected from the group comprising hypocrellins, cercosporin, phleichromes, elsinochromes, cladochromes, erythroaphins, and calphostins.

20. The composition of claim 18 wherein the perylenequinone is a derivative of hypocrellin A or hypocrellin B.

21. The composition of claim 19 wherein the perylenequinone is non-toxic at high concentrations in its non-activated state and toxic at low concentrations in its activated state.

22. The composition of claim 20 wherein the hypocrellin derivative is non-toxic at high concentrations in its non-activated state and toxic at low concentrations in its activated state.

23. The composition of claim 18 wherein the binding agent is a targeting agent for a disease, disorder, malady, or condition.

24. The composition of claim 18 wherein the binding agent is selected from the group consisting of an antibody, a monoclonal antibody, or a fragment thereof.

25. The composition of claim 24 wherein the binding agent is an antibody that binds to an epitope of a cancer cell, said cancer cell is selected from the group consisting of ovarian cancer, breast cancer, and gastrointestinal cancer.

26. The composition of claim 18 wherein the binding agent binds to a DNA minor groove.

27. A composition comprising a hypocrellin derivative conjugated to a binding agent.

28. The composition of claim 27 wherein the hypocrellin derivative is non-toxic at high concentrations in its non-activated state and toxic at low concentrations in its activated state.

29. The composition of claim 27 wherein the binding agent is a targeting agent for a disease, disorder, malady, or condition.

30. The composition of claim 27 wherein the binding agent is selected from the group consisting of an antibody, a monoclonal antibody, or a fragment thereof.

31. The composition of claim 27 wherein the binding agent is an antibody that binds to an antigen of a cancer cell, said cancer cell is selected from the group consisting of ovarian cancer, breast cancer, and gastrointestinal cancer.

32. The composition of claim 27 wherein the binding agent binds to a DNA minor groove.

33. The composition of claim 27 wherein the hypocrellin derivative is a derivative of hypocrellin B.

34. A composition comprising a derivative of hypocrellin B conjugated to a binding agent, said binding agent selected from the group consisting of an antibody, a

monoclonal antibody, or a fragment thereof; said binding agent binds to an antigen of a cancer cell, said cancer cell is selected from the group consisting of ovarian cancer, breast cancer, and gastrointestinal cancer; and wherein said derivative of hypocrellin B is a functionalized derivative that can be activated by exposing the derivative to a pre-determined wavelength of light.

35. The method of claim 1 wherein the method of treatment comprises treating skin conditions, cancer, viral diseases, retroviral diseases, bacterial diseases, and fungal diseases.

36. The method of claim 1 wherein the predetermined wavelength is between about 400 nm and about 850 nm.

37. The method of claim 36 wherein the predetermined wavelength is between about 600 nm and about 700 nm.

38. A method for destroying or inactivating tumor cells comprising administering a suitable amount of at least one hypocrellin derivative, and activating the hypocrellin derivative.

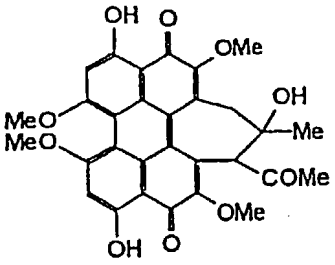
39. The method of claim 38 wherein activating the hypocrellin derivative comprises exposing the hypocrellin derivative to a predetermined wavelength of light.

40. A method for treating a disease or condition comprising administering a composition comprising a hypocrellin derivative and at least one of a pKa modifier, a buffer, a salt, a base, an acid, saline, and an adjuvant.

Me
COMe
OMe
OMe
HNR
O
HNR
O
MeO
MeO
Isomer B
+
Me
COMe
OMe
OMe
O
O
RNH
RNH
MeO
MeO
Isomer A
RNH2
Me
COMe
OMe
OMe
O
O
OH
OH
MeO
MeO
Hypocrellin B

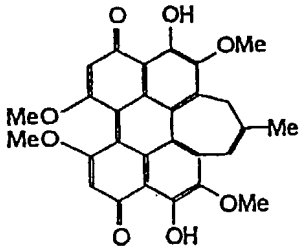
Figure 1:

Figure 2: Chemical Structures



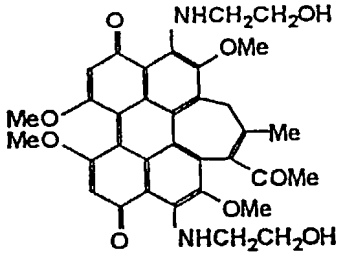
1

HYPOCRELLIN A (HA)



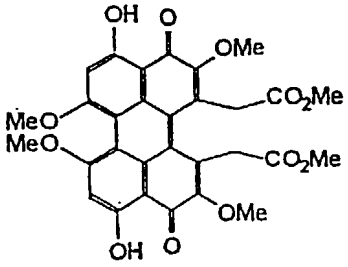
2

DAHA



3

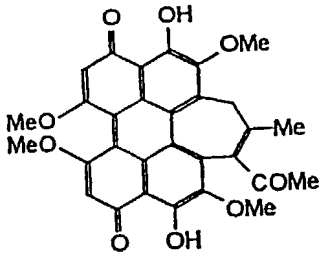
HBEA-R1



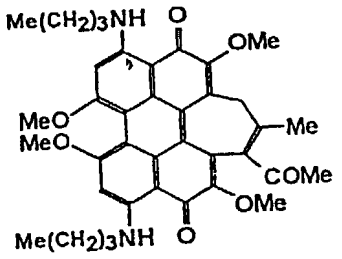
4

JL-1-1

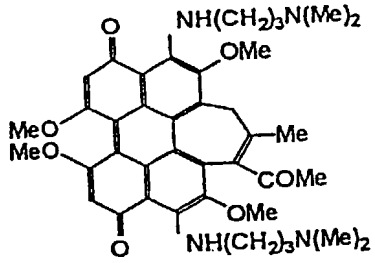
Figure 2: (continued): Chemical Structures



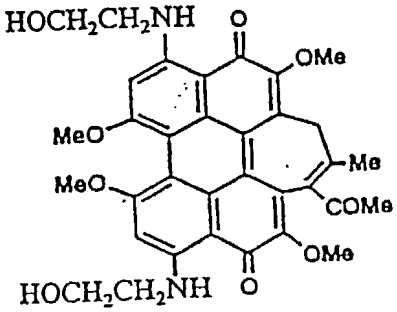
HYPOCRELLIN B (HB) 5



HBBA-R2 6

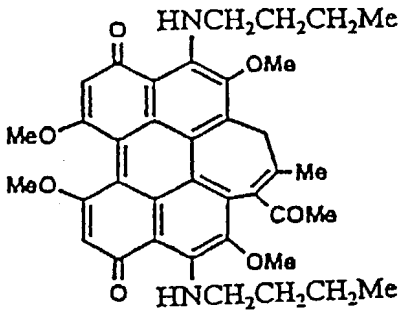


HBDP-R1 7



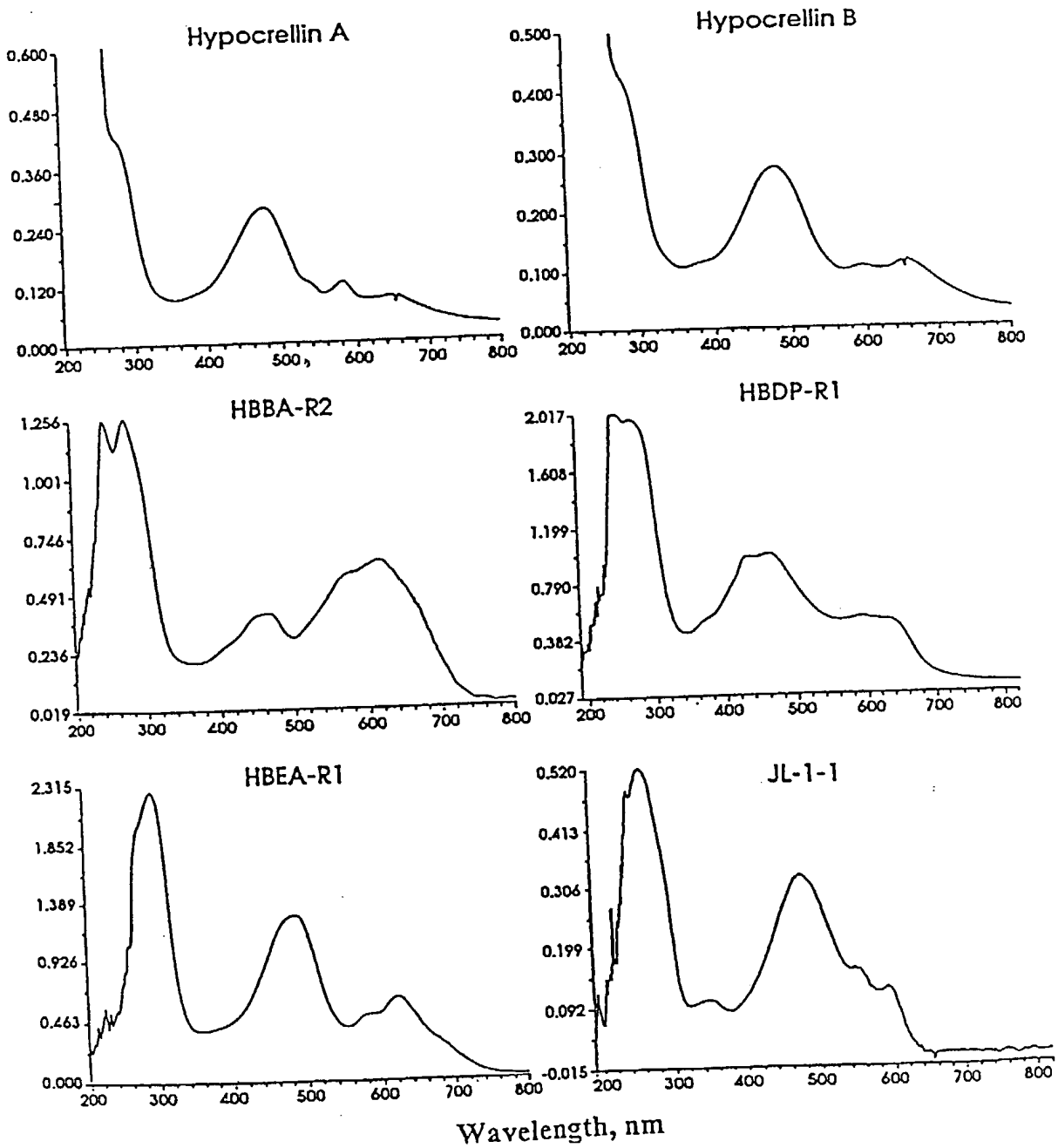
Compound 3A 8

Figure 2 (continued): Chemical Structures



Compound 4B

Figure 3:


Hypocrellin A
Hypocrellin B
HBBA-R2
HBDP-R1
HBEA-R1
JL-1-1
Wavelength, nm

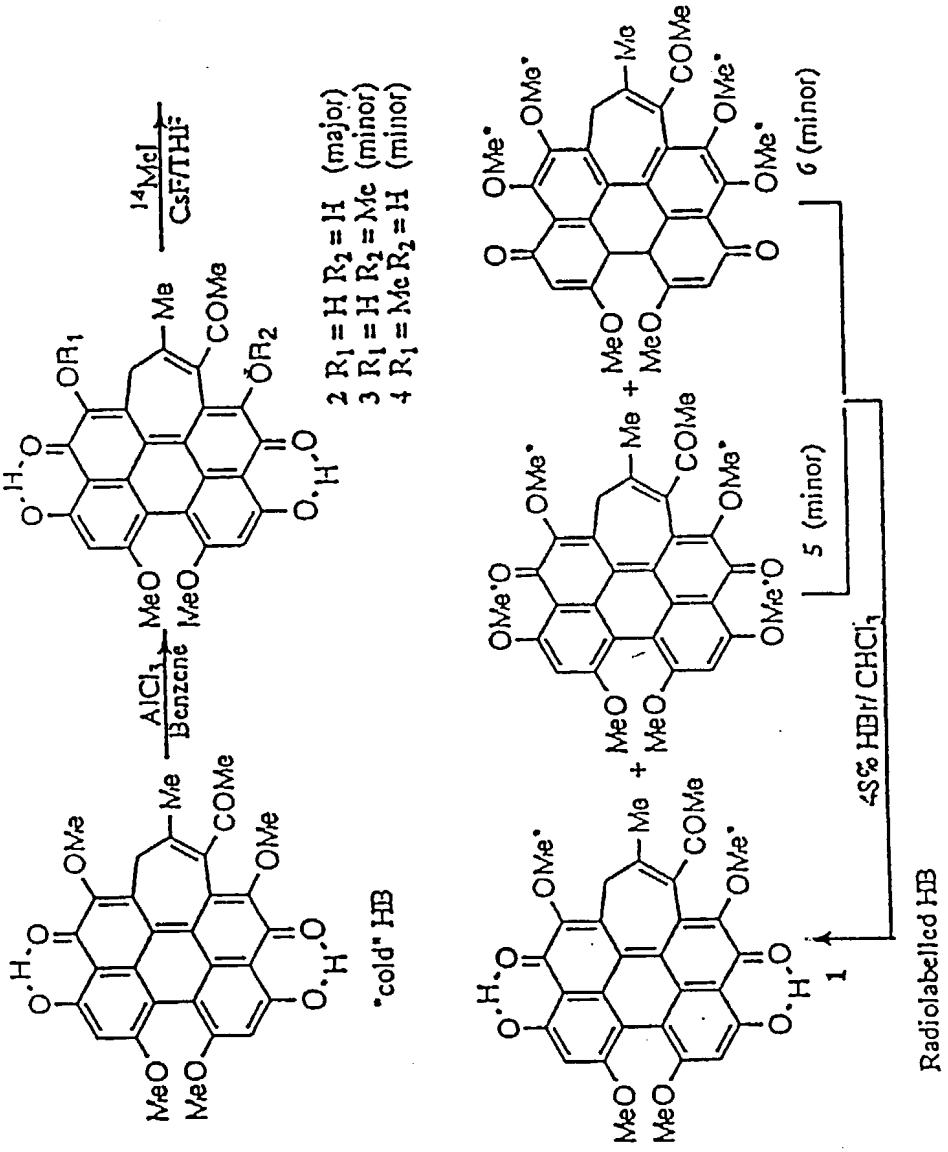

AlCl3
Benzene
MeO
MeO
Me
COMe
OMe
OR1
OR2
14MeI
CsF/THF
2 R1 = H R2 = H (major)
3 R1 = H R2 = Me (minor)
4 R1 = Me R2 = H (minor)
"cold" HB
45% HBr/CHCl3
1
5 (minor)
6 (minor)
Radiolabelled HB
Me* = 14C-labelled

Figure 4:

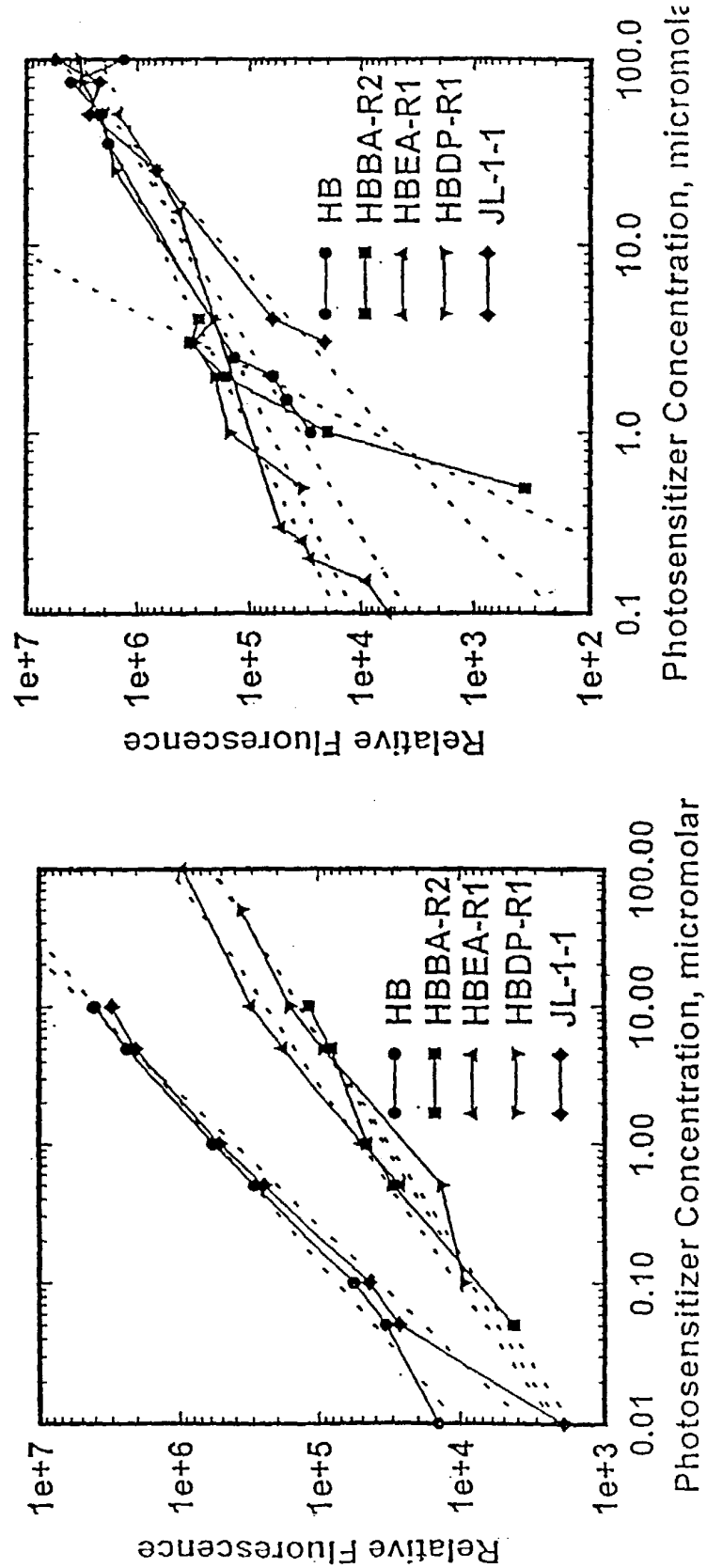
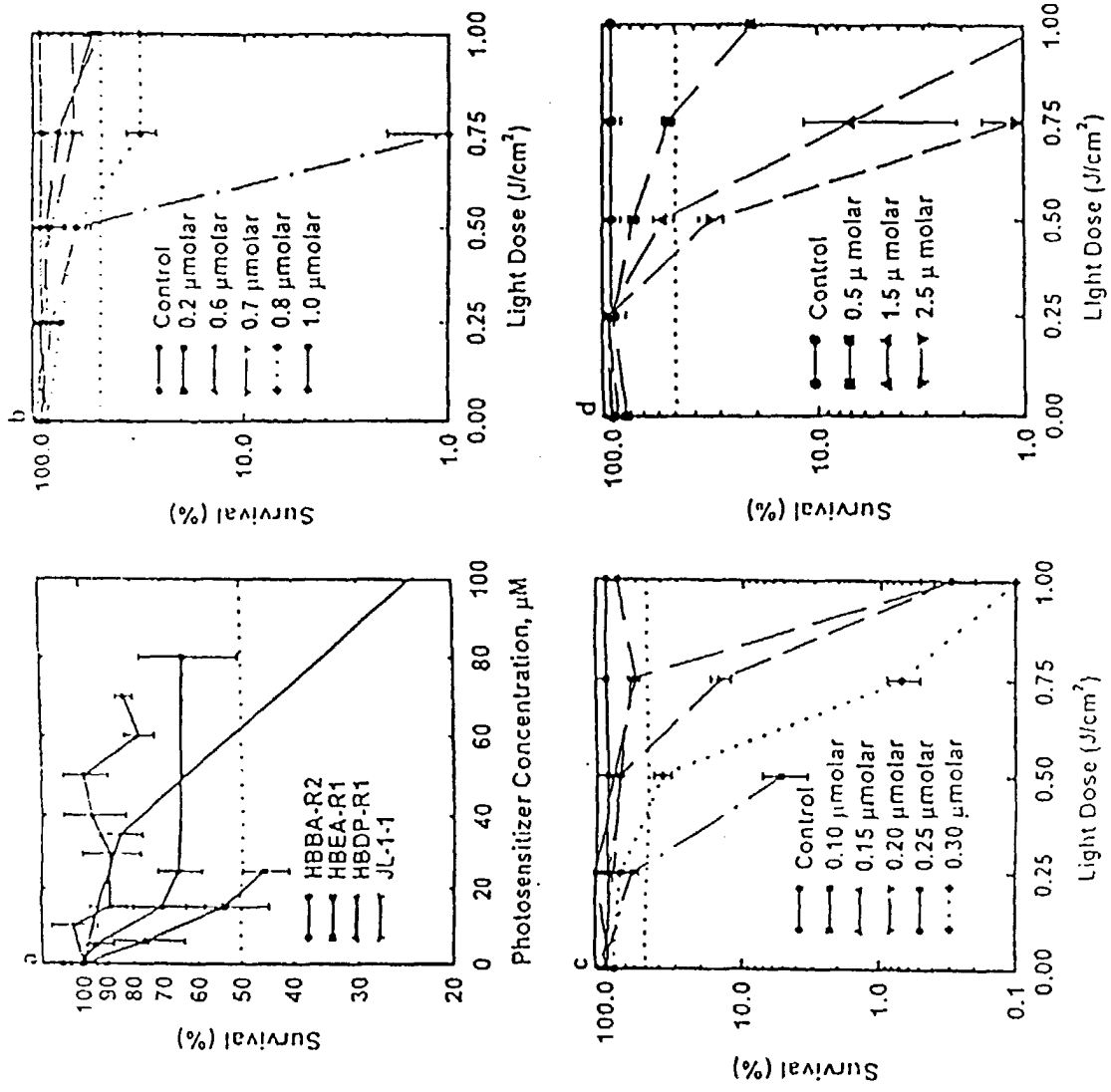
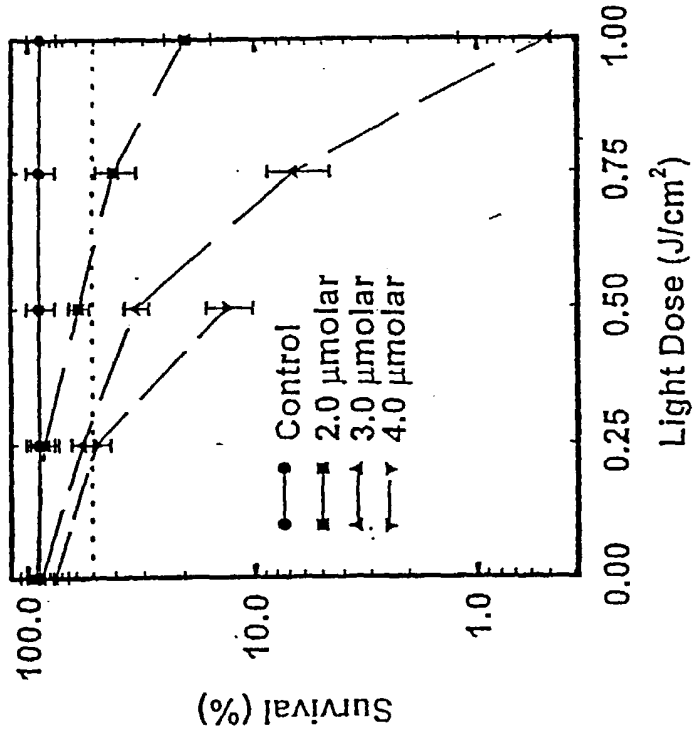

a.
Relative Fluorescence
1e+7
1e+6
1e+5
1e+4
1e+3
0.01
0.10
1.00
10.00
100.00
Photosensitizer Concentration, micromolar
HB
HBBA-R2
HBEA-R1
HBDP-R1
JL-1-1
b.
Relative Fluorescence
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
0.1
1.0
10.0
100.0
Photosensitizer Concentration, micromola
HB
HBBA-R2
HBEA-R1
HBDP-R1
JL-1-1

Figure 5:

FIGURE 6


b
Control
0.2 µmolar
0.6 µmolar
0.7 µmolar
0.8 µmolar
1.0 µmolar
Survival (%)
Light Dose (J/cm²)
d
Control
0.5 µ molar
1.5 µ molar
2.5 µ molar
Survival (%)
Light Dose (J/cm²)
a
HBBA-R2
HBEA-R1
HBDP-R1
JL-1-1
Survival (%)
Photosensitizer Concentration, µM
c
Control
0.10 µmolar
0.15 µmolar
0.20 µmolar
0.25 µmolar
0.30 µmolar
Survival (%)
Light Dose (J/cm²)


Survival (%)
100.0
10.0
1.0
0.00
0.25
0.50
0.75
1.00
Light Dose (J/cm²)
Control
2.0 µmolar
3.0 µmolar
4.0 µmolar

c.

Figure 6:

Figure 7:

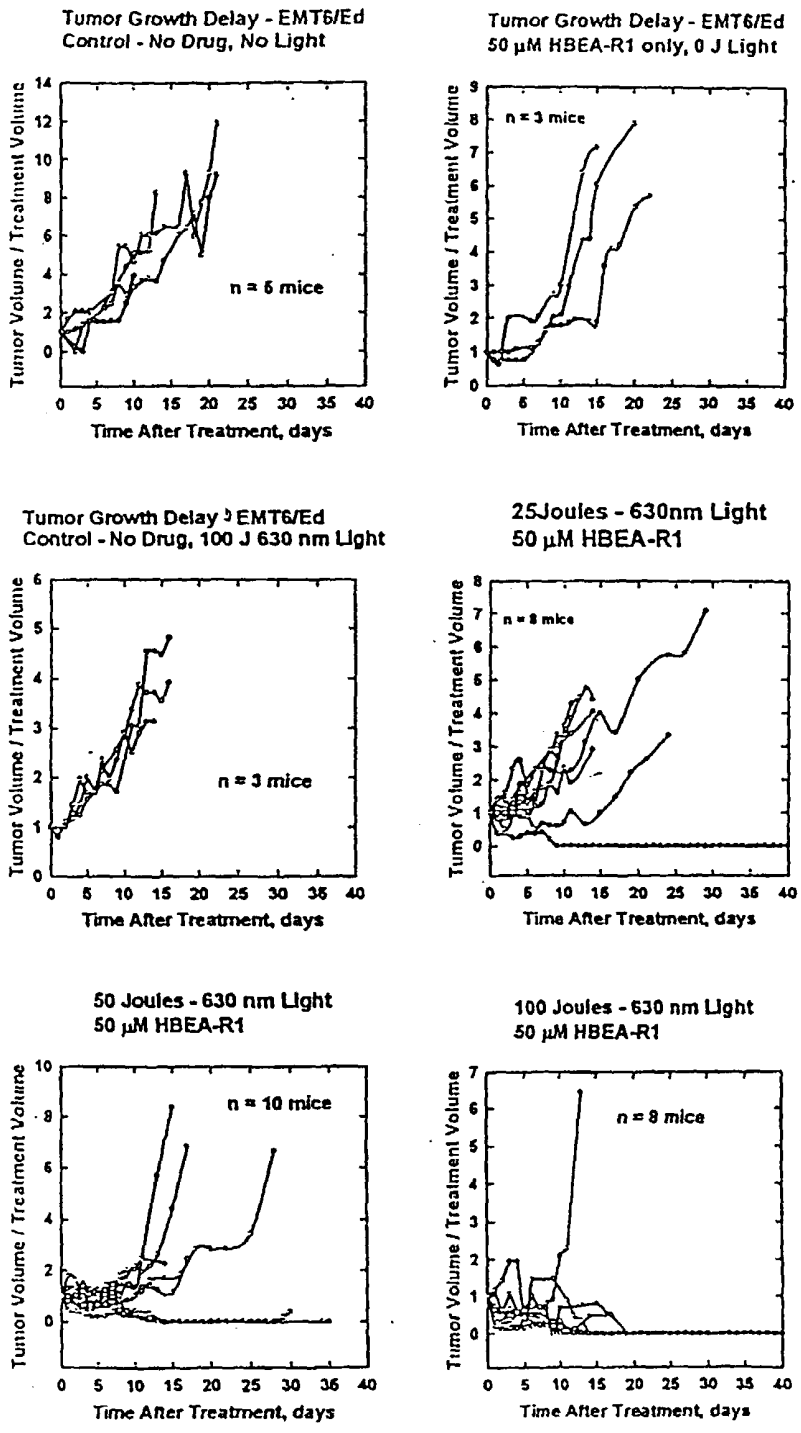

Tumor Growth Delay - EMT6/Ed
Control - No Drug, No Light
n = 6 mice
Tumor Growth Delay - EMT6/Ed
50 µM HBEA-R1 only, 0 J Light
n = 3 mice
Tumor Growth Delay - EMT6/Ed
Control - No Drug, 100 J 630 nm Light
n = 3 mice
25Joules - 630nm Light
50 µM HBEA-R1
n = 8 mice
50 Joules - 630 nm Light
50 µM HBEA-R1
n = 10 mice
100 Joules - 630 nm Light
50 µM HBEA-R1
n = 8 mice
Tumor Volume / Treatment Volume
Time After Treatment, days

Figure 8:

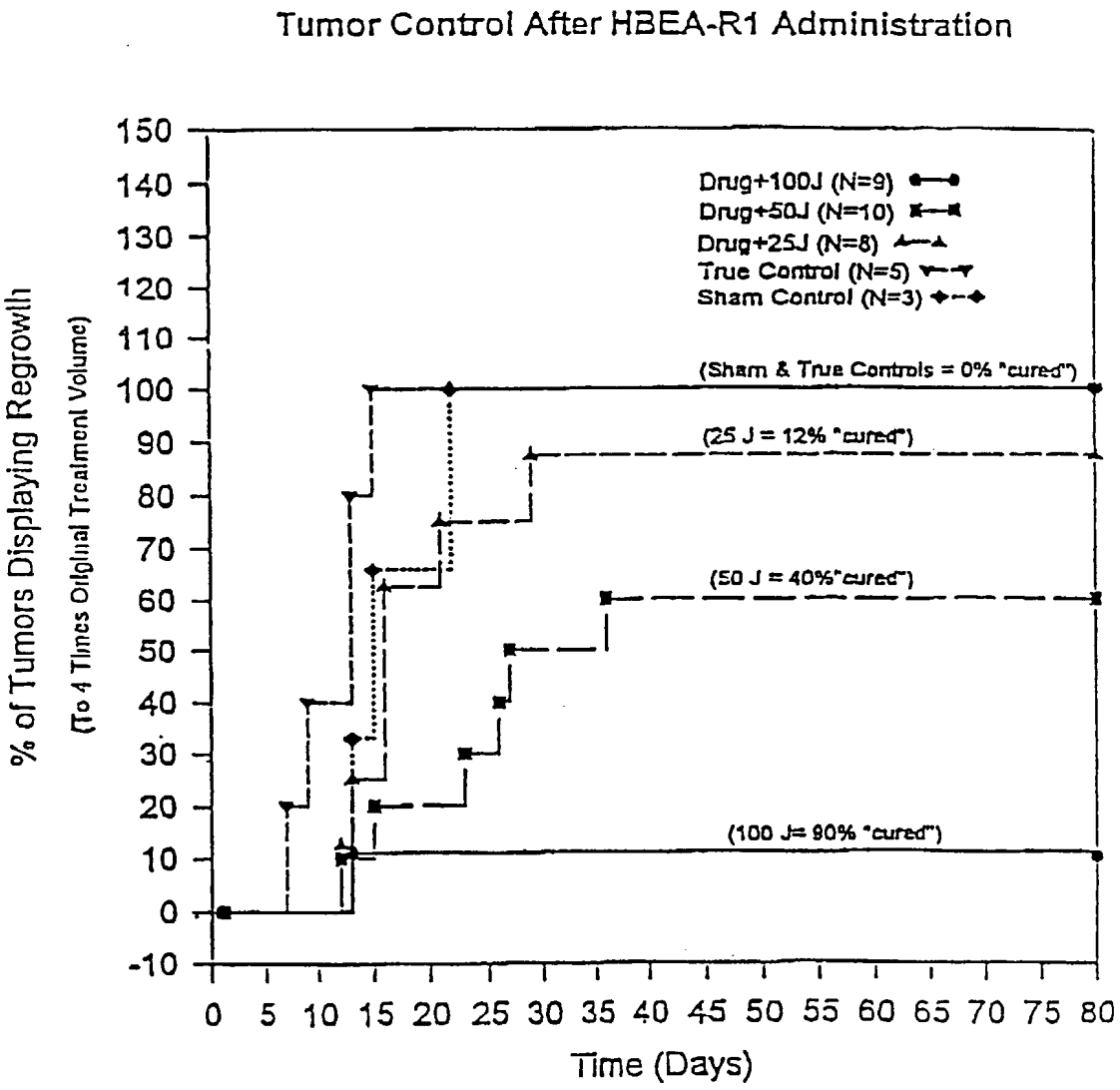
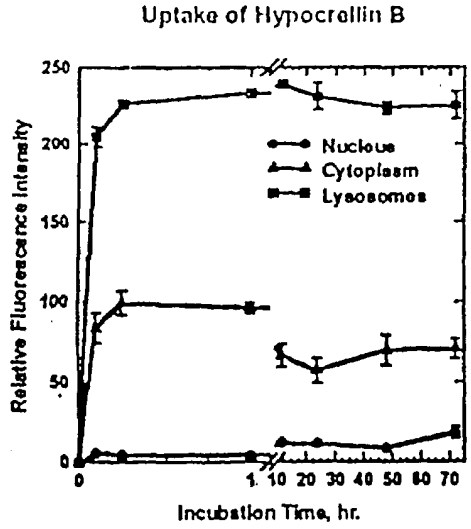
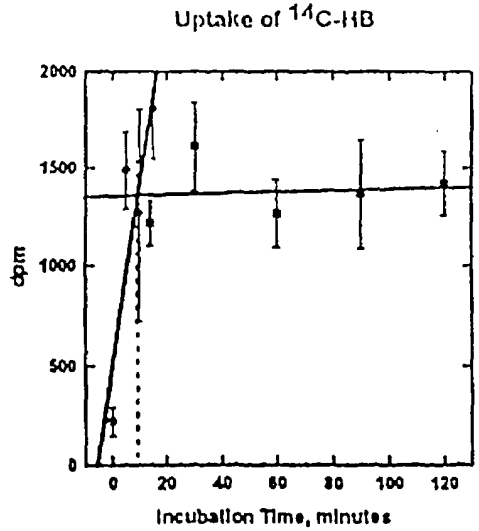

Tumor Control After HBEA-R1 Administration
% of Tumors Displaying Regrowth
(To 4 Times Original Treatment Volume)
Drug+100J (N=9)
Drug+50J (N=10)
Drug+25J (N=8)
True Control (N=5)
Sham Control (N=3)
(Sham & True Controls = 0% "cured")
(25 J = 12% "cured")
(50 J = 40% "cured")
(100 J= 90% "cured")
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
Time (Days)

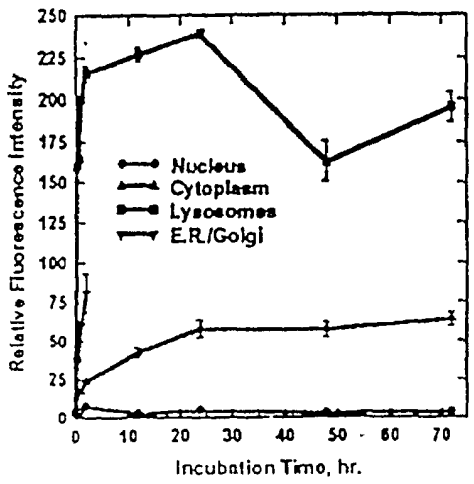
Figure 9:



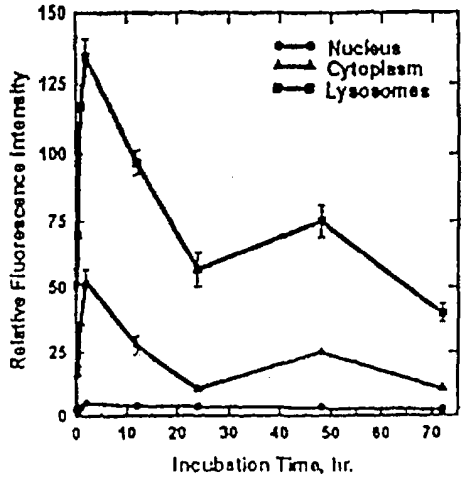
a.



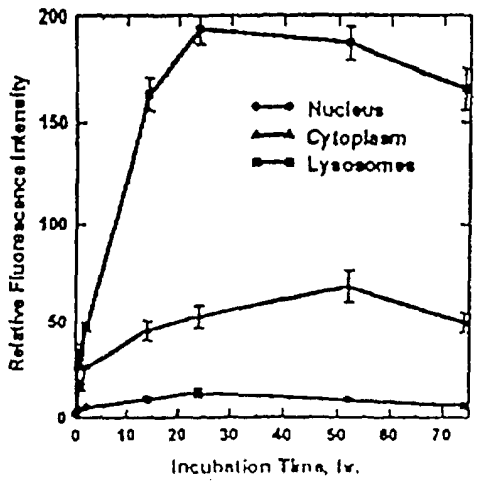
b.



c.

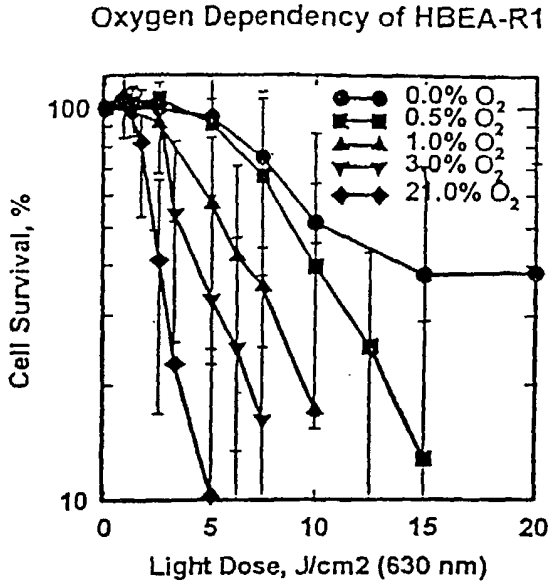


d.



e.

Figure 10:


Oxygen Dependency of HBEA-R1
Cell Survival, %
100
10
0
5
10
15
20
Light Dose, J/cm2 (630 nm)
0.0% O2
0.5% O2
1.0% O2
3.0% O2
21.0% O2

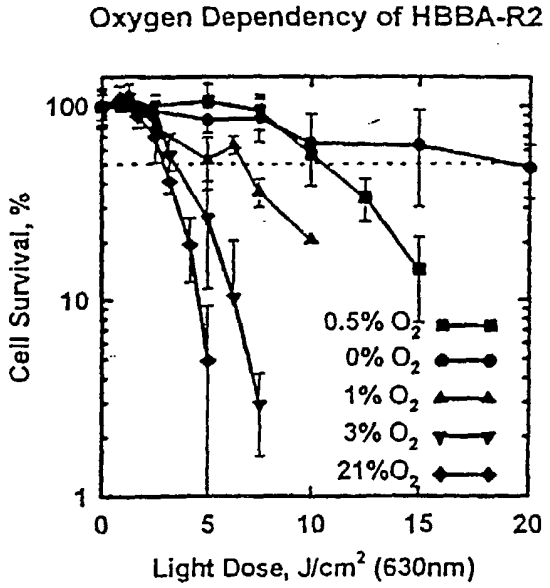

Oxygen Dependency of HBBA-R2
Cell Survival, %
100
10
1
0
5
10
15
20
Light Dose, J/cm2 (630nm)
0.5% O2
0% O2
1% O2
3% O2
21%O2

Figure 11:

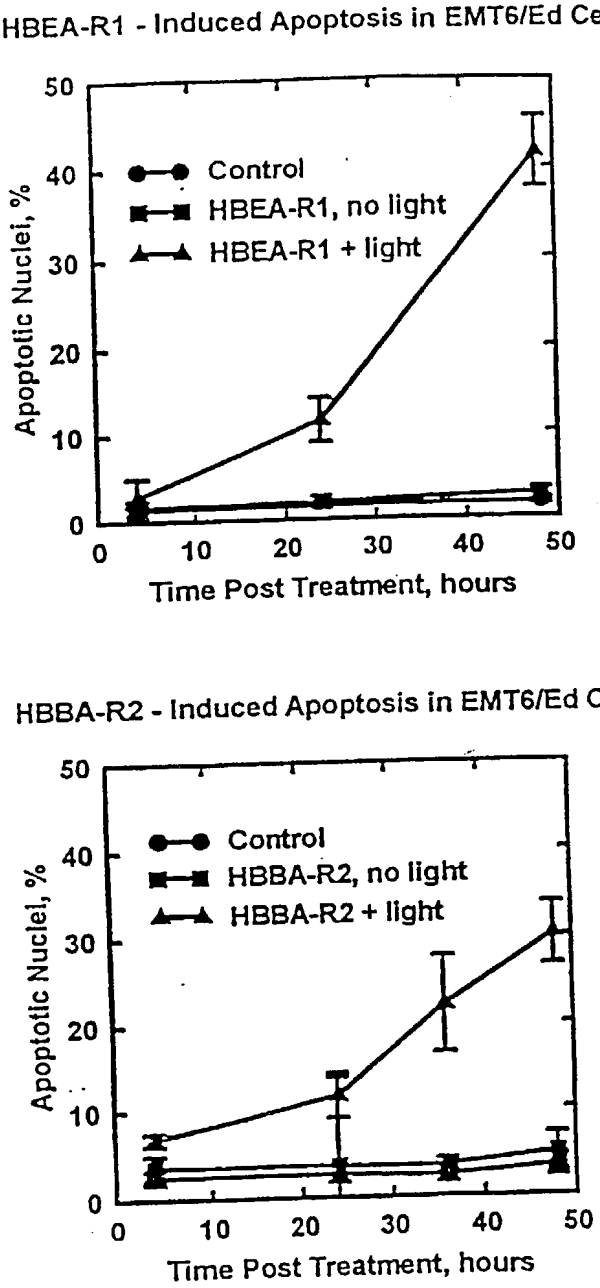
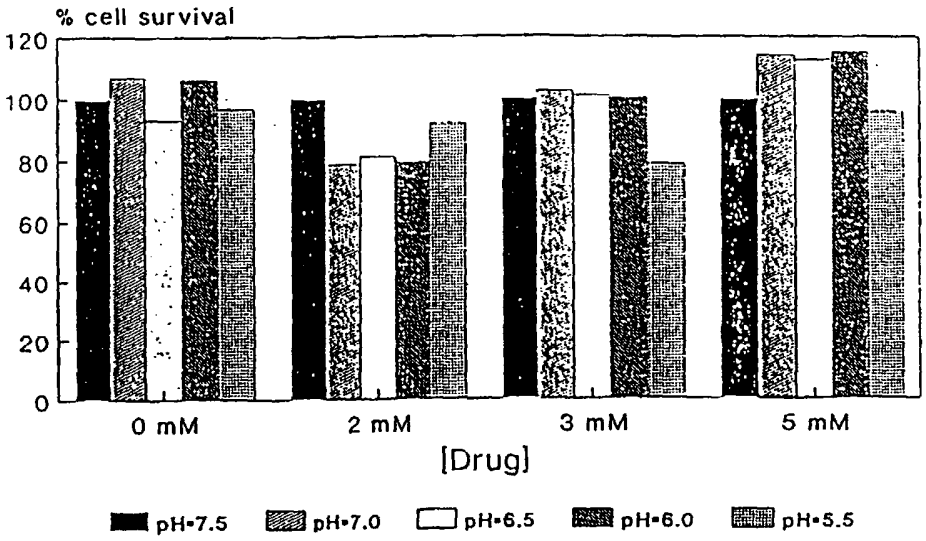

HBEA-R1 - Induced Apoptosis in EMT6/Ed Cells
Apoptotic Nuclei, %
Time Post Treatment, hours
Control
HBEA-R1, no light
HBEA-R1 + light
HBBA-R2 - Induced Apoptosis in EMT6/Ed Cells
Apoptotic Nuclei, %
Time Post Treatment, hours
Control
HBBA-R2, no light
HBBA-R2 + light

Figure 12.

a.


pH Effect on HA
Subjected to 630 nm light
% cell survival
120
100
80
60
40
20
0
0 mM
2 mM
3 mM
5 mM
[Drug]
pH=7.5
pH=7.0
pH=6.5
pH=6.0
pH=5.5

b.

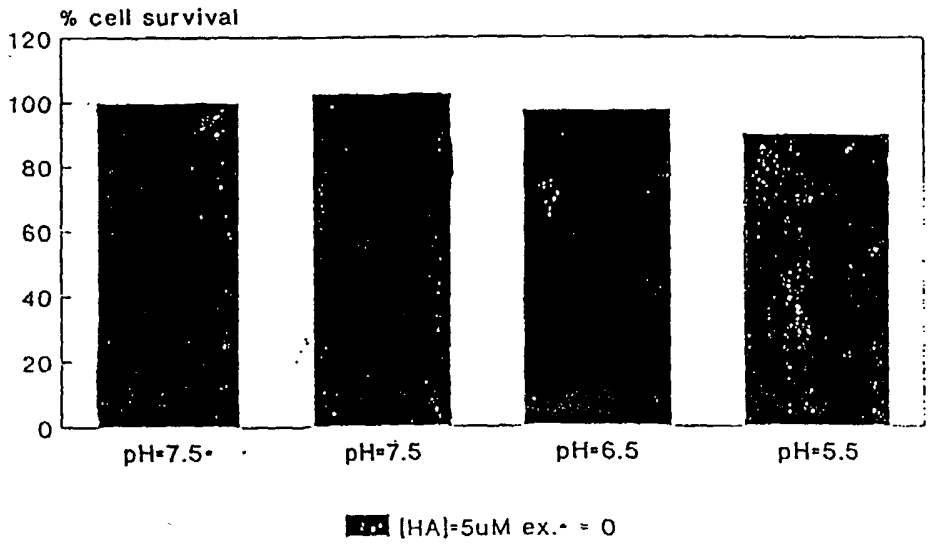
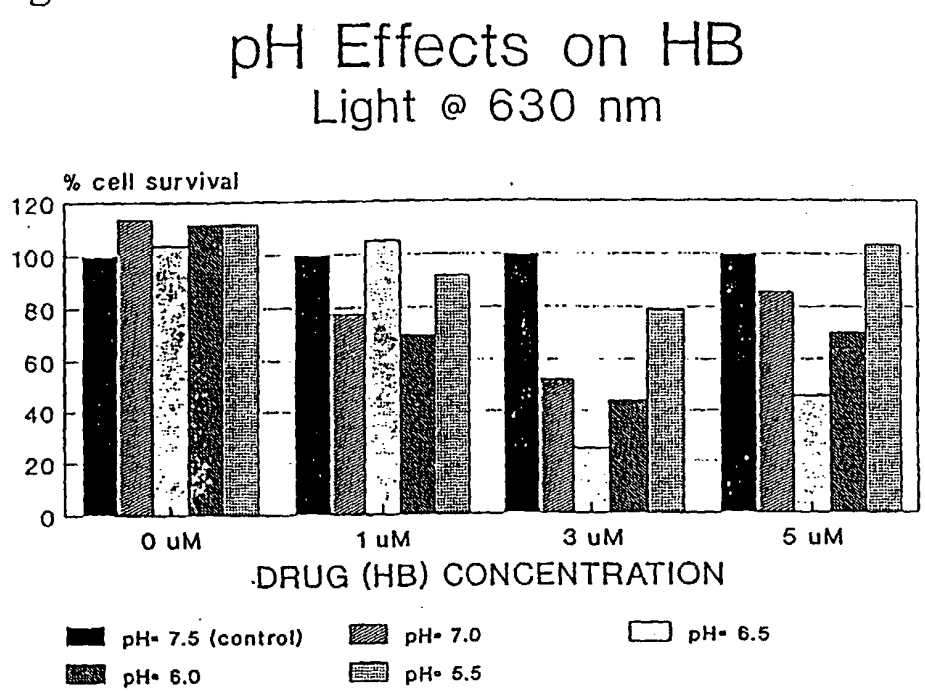
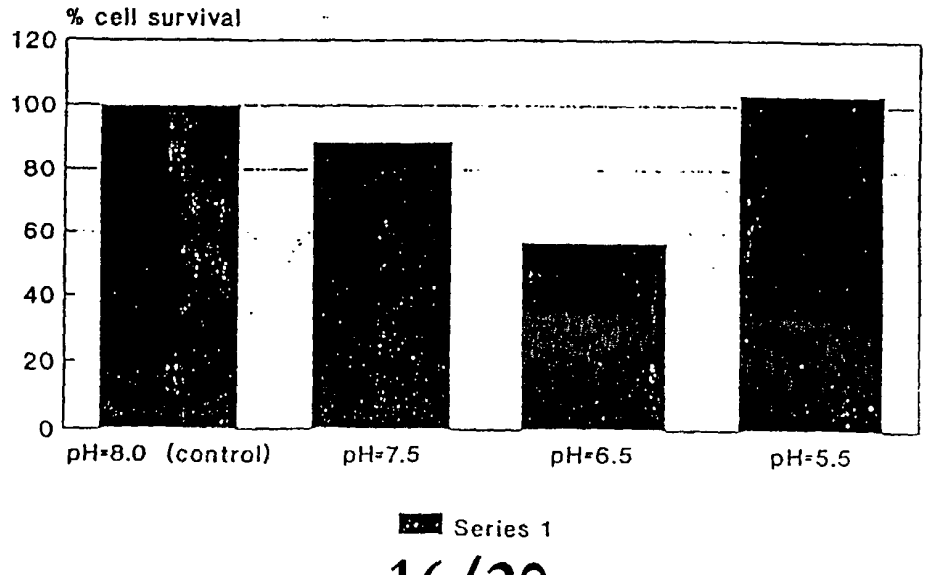

pH Effects on HA
Dark Toxicity
% cell survival
120
100
80
60
40
20
0
pH=7.5
pH=7.5
pH=6.5
pH=5.5
[HA]=5uM ex. = 0

Figure 12. (CONT)

c.

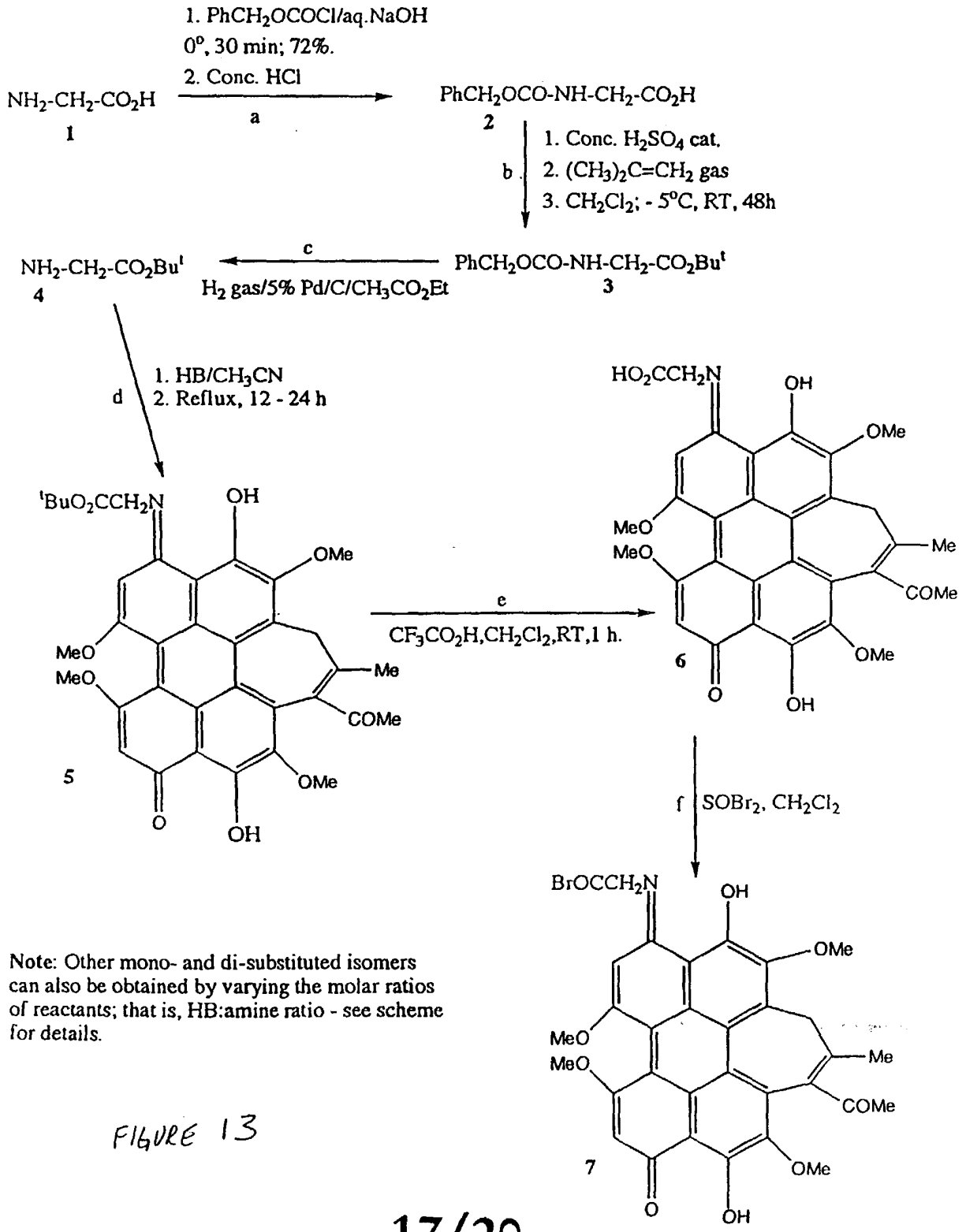


d.



[HB]=5uM

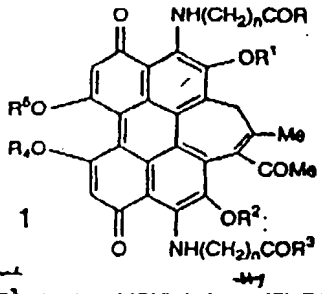
SYNTHESIS OF AMINO ACID DERIVATIVES OF HYPOCRELLIN B



Note: Other mono- and di-substituted isomers can also be obtained by varying the molar ratios of reactants; that is, HB:amine ratio - see scheme for details.

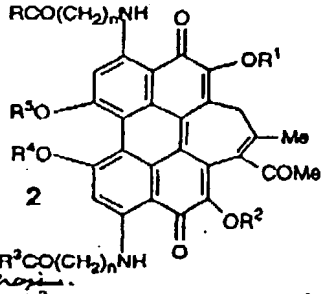
FIGURE 13

FURTHER AMINO DERIVATIVES OF HYPOCRELLIN B



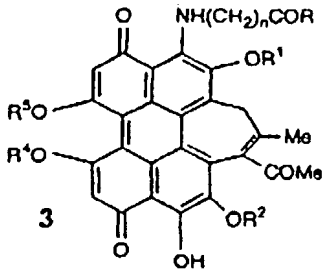
1

R, R^3 = Hydroxyl (OH); halogen (Cl, Br),
R^1, R^2, R^4, R^5 = Alkyl (C_1 - C_6)
n = 1 - 5



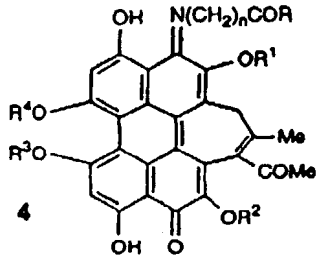
2

R, R^3 = Hydroxyl (OH); halogen (Cl, Br)
R^1, R^2, R^4, R^5 = Alkyl (C_1 - C_6)
n = 1 - 5



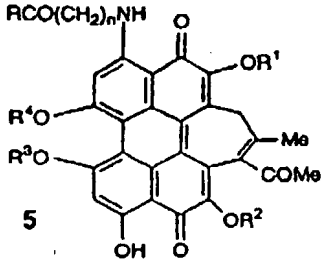
3

R = Hydroxyl (OH); halogen (Cl, Br)
R^1, R^2, R^3, R^4 = Alkyl (C_1 - C_6)
n = 1 - 5



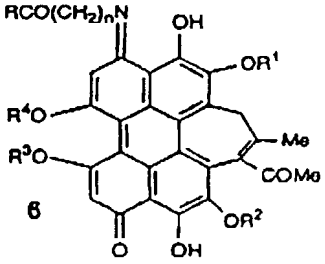
4

R = Hydroxyl (OH); halogen (Cl, Br)
R^1, R^2, R^3, R^4 = Alkyl (C_1 - C_6)
n = 1 - 5



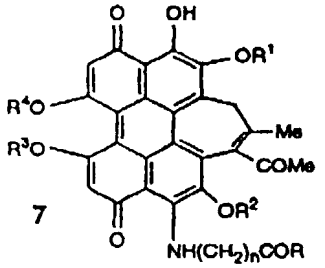
5

R = Hydroxyl (OH); halogen (Cl, Br)
R^1, R^2, R^3, R^4 = Alkyl (C_1 - C_6)
n = 1 - 5



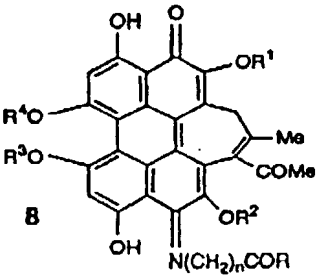
6

R = Hydroxyl (OH); halogen (Cl, Br)
R^1, R^2, R^3, R^4 = Alkyl (C_1 - C_6)
n = 1 - 5



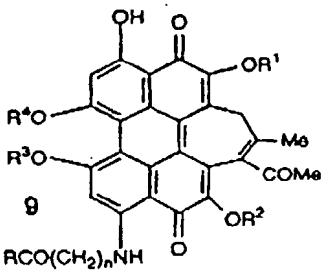
7

R = Hydroxyl (OH); halogen (Cl, Br)
R^1, R^2, R^3, R^4 = Alkyl (C_1 - C_6)
n = 1 - 5



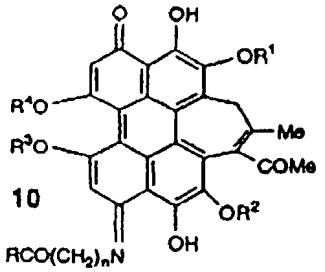
8

R = Hydroxyl (OH); halogen (Cl, Br)
R^1, R^2, R^3, R^4 = Alkyl (C_1 - C_6)
n = 1 - 5



9

R = Hydroxyl (OH); halogen (Cl, Br)
R^1, R^2, R^3, R^4 = Alkyl (C_1 - C_6)
n = 1 - 5

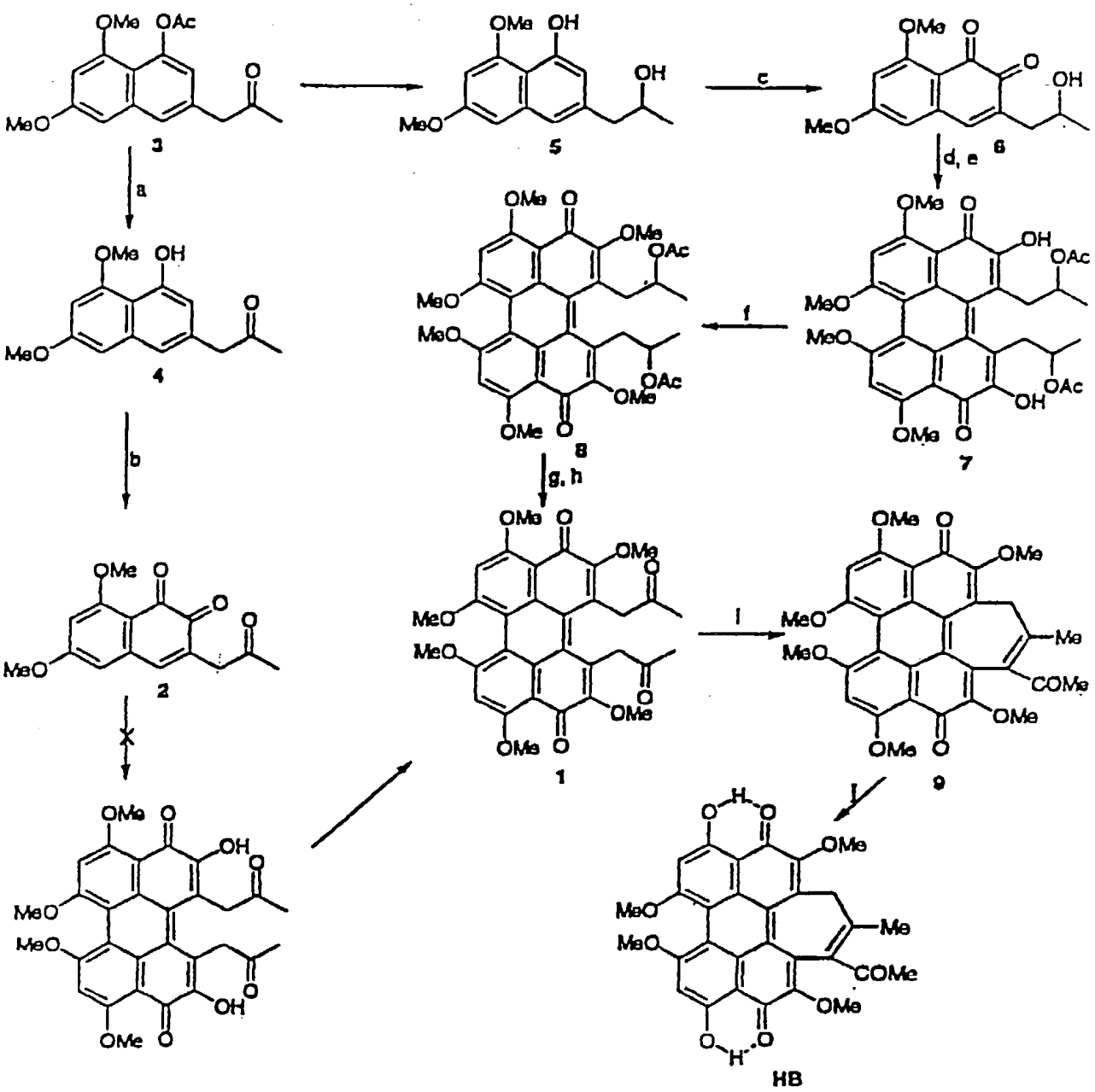


10

R = Hydroxyl (OH); halogen (Cl, Br)
R^1, R^2, R^3, R^4 = Alkyl (C_1 - C_6)
n = 1 - 5

FIGURE 14

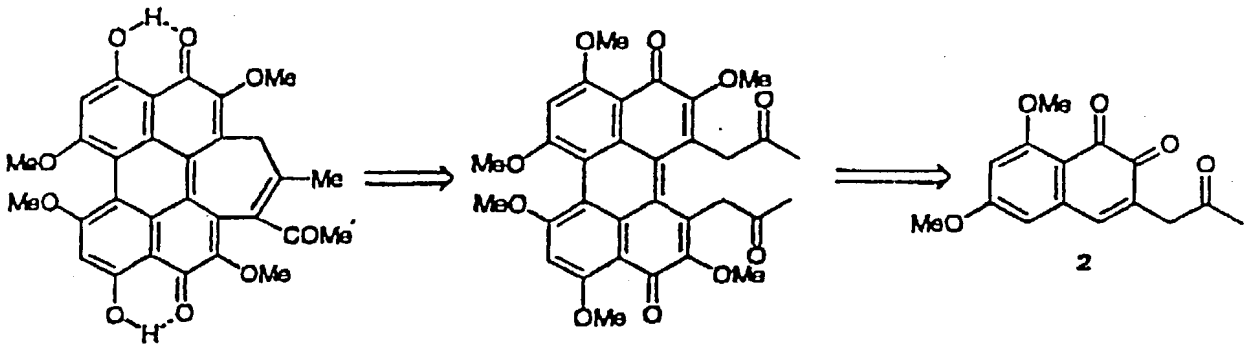
Scheme II



Reagents and conditions: (a) K_2CO_3, MeOH-H_2O, RT, 2 h; 84%; (b) $(PhSeO)_2O$, THF, 50°C, 20 min; 81%; (c) $(PhSeO)_2O$, THF, 50°C, 20 min; 87%; (d) Ac_2O, Py, DMAP (cat.), $CHCl_3$, RT, 3 h; 95%; (e) TFA then $FeCl_3$, RT, 2h; 88%; (f) MeI, CsF, THF, RT, 16 h; 84%; (g) K_2CO_3, MeOH-H_2O, RT, 12 h; 96%; (h) CrO_3-Py, CH_2Cl_2, RT, 1 min; 66%; (i) LiOH, MeOH-H_2O, RT, 1h; 85%; (j) 48% HBr, $CHCl_3$, RT, 1 h; 90%.

Figure 2/5

Scheme I



HB 1 2

Figure 16